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From the Institute of Physiology University of Uppsala, Uppsala, Sweden

An Analysis of the Current Voltage Relationship in Excitable Nitella Cells

By

Torsten Teorell

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Abstract

Teorell, T. *An analysis of the current-voltage relationship in excitable Nitella cells.* Acta physiol. scand. 1961. 53. 1—6. — Experiments were performed on cells of the fresh water algae *Nitella* using "triangular waves" of varying frequencies, i. e. depolarizing-hyperpolarizing currents with varying, linear slopes. With very low frequencies one obtains "steady state rectification" curves, but with intermediate frequencies metability phenomena are revealed. The experimental findings of "dynamic current-voltage" characteristics are compared with the theoretical results, which can be predicted from the author's "electrohydraulic" hypothesis for excitability phenomena. It is suggested that electro-osmotic processes, arising from presence of membrane fixed charges, may play a part in the excitability of *Nitella*.

In recent years it has become common to characterize the excitability properties of nerves and other tissues by measuring the changes in the transmembrane potential after application of hyperpolarizing or depolarizing currents. The results are usually plotted as current versus resulting voltage. Such a current-voltage relationship is also denoted "rectification curve" because the slopes to the curve define the tissue (slope) conductance (Cole and Curtis 1941). The usual finding is that the rectification curve indicates a marked increase in conductance during depolarization. For the single nerve fiber it is well known that there can be an apparent change between resting conductance and spike conductance of the order of 1 : 200. The nature of this marked change has been discussed in various terms as "delayed rectification etc." For a number of years the author has attempted to analyse the rectification

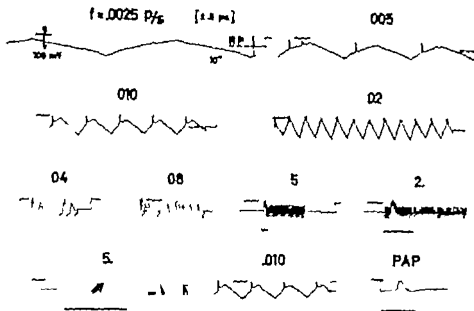


Fig. 1. A *Nitella* cell subject to triangular current waves of varying frequencies. Peak current amplitudes ± 0.5 μA. Horizontal bars indicate zero potential. Dashed bars, marked *RP* indicate resting potential. Time bars represent 10 sec. *PAP* shows a propagated action potential following the experiment.

curves in terms of an "electrohydraulic" theory in which electro-osmosis has a prominent part (Teorell 1958 a, 1959 a, b, c, 1960). In a paper by Teorell (1958 b) an attempt was made to investigate the rectification in a plant cell (*Nitella*) in relation to the electro-osmosis. The results indicated at least a formal validity of the electrohydraulic hypothesis. Since then we have performed numerous investigations on the *Nitella* with various methods in order to characterize the excitability mechanism. Especially useful we have found the application of triangular current waves of different frequencies. With very slow waves (of a frequency of the order of 0.002 p/s) it was possible to obtain a current voltage relation curve, which was smooth and almost without hysteresis and without any appearance of "action potential" responses (the resulting plot is in shape similar to that marked *Q* in Fig. 2 b). In the meantime Findlay (1959) has published a current voltage relation for the *Nitella*, which has a similar shape although without attempts to a closer analysis of its nature.

Experiments on the current-voltage characteristics of the Nitella. The steady state method of using depolarisation and hyperpolarisation currents as employed by for instance Findlay gives meager information about the possible intrinsic nature of the resulting rectification curve. More information could be ob-

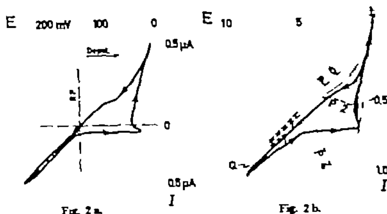


Fig. 2 a. Fig. 2 b.

Fig. 2 a. A current-voltage record of Nitella with triangle wave of f = 0.010 p/s. RP = resting potential (before addition of NaCl).

Fig. 2 b. A reconstructed current-voltage relation according to the "electrohydraulic" theory. See text for explanation.

tained by investigation of the non-steady state behaviour which reveals interesting details of the dynamic properties of the excitable system. We have therefore employed "triangular" depolarisation-hyperpolarisation current waves of a frequency which discloses otherwise "hidden" instability phenomena.

Methods

The experimental technique was essentially the same as described previously (Teorell 1958 b) with the exception that the square wave generator was substituted by a triangular wave generator. The frequency was varied in the range of 0.0025 p/s to 5.0 p/s. The peak current values were + 0.5 and — 0.5 microamps corresponding to current density of about 5 microamps/cm. One current electrode was placed in pool of pond water and another in 0.1 M KCl, i.e. the essential changes observed took place under the pond water electrode.

Results

A typical result is depicted in Fig. 1 and shows that potential spikes are elicited at an intermediate frequency range of about f = 0.01 p/s. The non-linear voltage response is especially evident at f = 0.0025 and 0.005 p/s. An interesting feature is also offered by the very high frequency response at 0.5 and 2.0 p/s, however these results will not be discussed in this paper.

The next step in the experimental analysis was to select the "dynamic" f = 0.01 condition and by means of a X-Y plotter obtain the current voltage relationship. Such a result is depicted in Fig. 2 a. In this particular experiment, however, some NaCl was added to the pond water in order to obtain an outside electrolyte concentration suitable for comparison with the theoretical analysis to follow (0.02 molarity of NaCl was used). This has also the added advantage of making the cell more excitable or even inducing spontaneous

firing The nature of this NaCl-stimulation will be discussed in a forthcoming paper The *I E* characteristic of Fig 2 a is by no means a single curve i e it displays marked "hysteresis An abrupt change takes place during the depolarisation phase close to the resting potential a sudden fl p over" is noticed. During the repolarizing return trace there is also a sudden brief transition in the voltage.

A theoretical analysis of the dynamic current-voltage relation. The following interpretation of the observed events is based essentially on the same concepts emerging from the electro-osmotic membrane oscillator which were used already in the previous square wave analysis" of the *Nitella* (TEORELL 1958 b) However an additional process allowing for membrane leakage has been added as described in the formalism given by TEORELL (1959 c, 1960)

The numerical solution was carried out by the analog computer technique according to the principles described by TEORELL (1960) The results are obtained directly as graphs (*e g* Fig. 2 b) The following numerical values were used in the machine solution of the electrohydraulic excitability equations s (hydraulic permeability coefficient) = 1 0 σ (leakage coefficient) = 1.6 l (electro-osmotic permeabil ty coefficient) = 1.5 and further coefficients were set as L = 40, q = 0.03 and an add tional I_E decrement of 10 units was added to allow for the effect on the water drag exerted by the external salt. The remaining parameters were exactly the same as described by TEORELL (1959 b, 1960) The machine frequency of the employed triangle wave was 0.5 p/s. The results obtained are given in arbitrary machine units and the zero current is displaced to the origin. The curves for constant (clamped") hydrostatic pressure marked *P* (dashed lines) as well as the steady stat rectification curve marked *Q* (dotted line) were obtained by appropriate machine operations.

The theoretical reconstruction result is depicted in Fig 2 b The dynamic path (the solid line with direction arrows) clearly shows a marked similarity with the experimental *Nitella* result shown in Fig 2 a The similarity appears to be so close in its essential features, that a detailed discussion of its significance seems worth while

Discussions on the nature of the current voltage characteristic — In the electrohydraulic excitability theory the state variables are the transmembrane potential (*E*) the membrane current (*I*) and the hydrostatic pressure difference (*P*) across the excitable membrane. The pressure factor is the resultant of the superposition of electrochemical and electro-osmotic driving forces (full discussions are given by TEORELL 1959 b)

According to this hypothesis an action potential or a continuous change in the polarisation state created by current flow should produce concomitant changes in pressure (and water flow) A full presentation of the interrelations between these three state variables is most rationally obtained by a three-dimensional phase space representation. Such an *I E P* representation can be found in another paper by the author (1961) The two-dimensional *I E* phase plot is accordingly somewhat deficient as it does not immediately reveal the influence of the pressure variable. However in the Fig 2 b some

steady state I-E graphs are given for a few constant P-values (as dashed lines). These "P-clamped" graphs should be visualized in planes parallel with the page sheets, as a third dimension perpendicular to the paper plane. The two P-clamps with $P > 0$ have a marked N-shaped course and are of a so called "dynatron" character (cf. FRANK 1956; FITZHUGH 1960), a concept often used in modern neurophysiology analysis of the "bistability" properties of, for instance, the nerve axon (cf. SPYROPOULOS and TASAKI 1960). It is characteristic of the dynatron-curves that they have a negative slope in the intermediate part, which signifies negative conductance. On the other hand, it is known from oscillation theory that the presence of negative conductances may lead to instability phenomena and oscillations. A closer analysis of the dynamic current voltage path in Fig. 2 b reveals that it extends in the three dimensions (in the figure the brief vertical lines connect the pressure graphs with the corresponding points of the path). The sudden transitions, or flip overs, of the I-E path belong actually to the instability domains of numerous dynatron curves. It can also be shown that the spontaneous, or induced, action potentials can be described in terms of a three-dimensional variation in the current-voltage-pressure space.

It may be added, that the computer analysis also quite faithfully predicts the frequency dependence of the triangular wave responses of the *Nitella* as presented in Fig. 1. In particular it should be pointed out that the very low frequency, i.e. the steady state I-E response, as depicted by the dotted line marked Q in Fig. 2 b, is similar to the one published by FINDLAY and also obtained by us.

The demonstration that the dynamic I-E-characteristic is a projection in a two-dimensional plane obtained from a three-dimensional I-E-P-space, of course, does not prove that the pressure variable is of importance in the living *Nitella* cell. But in any case, the electrohydraulic theory has been able to give a reasonable understanding of the formalism of the observed phenomena in terms of well defined quantities. In this paper we have confined the discussions entirely to a theory based on physical state variables. This analysis does not preclude the possibility that ionic events, for instance in terms of potassium and sodium-exchange, may be included in the above formalism, or may offer alternative explanations to the findings. The basic assumption of the presence of fixed charges, i.e. immobile ionic groups in the submicroscopic architecture of the excitable tissue structures offers many possibilities for understanding the mechanism of ion and water transport. It is hoped that further work will elucidate possible relations between the physical events and these membrane transport phenomena.

The costs of the equipment used in this work have been defrayed by grants from The Swedish Medical Research Council, The Swedish Cancer Association and the U. S. Air Research and Development Command, Grant No. AF 61 (052)—363, which are gratefully acknowledged. — My thanks are also due to Miss Eivor Hellstrand for valuable assistance throughout the work.

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From the Department of Physiology, University of Göteborg, Göteborg, Sweden

The Range of Effect of the Sympathetic Vasodilator Fibres with Regard to Consecutive Sections of the Muscle Vessels

By

Björn Folkow, Stefan Mellander and Bengt Öberg

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Abstract

Folkow, B., S. Mellander and B. Öberg. *The range of effect of the sympathetic vasodilator fibres with regard to consecutive sections of the muscle vessels.* Acta physiol. scand. 1961. 53. 7—22. — The effect of the activation of the sympathetic cholinergic vasodilator fibres has been studied by a technique permitting an analysis of the reactions in the pre- and postcapillary resistance vessels and the capacitance vessels of a skeletal muscle region. The experiments indicate that the dilator fibres are distributed essentially to the bigger precapillary resistance vessels of the muscles, where they can induce almost maximal dilatation, while it is likely that other consecutive vascular sections more or less completely lack such an innervation. The data do not support the view that the dilator fibres open up non-nutritional specific 'shunt vessels', but the above-mentioned fibre distribution may in certain experimental situations create a 'functional shunting' by causing an uneven distribution of capillary flow. A central excitation of these fibres in alarm situations, such as has been suggested, would thus mean an immediate massive blood flow increase in the skeletal muscles, with little or no regional blood 'pooling' or opening up of closed capillaries. However, as soon as the skeletal muscles are brought into action, the local production of vasodilator metabolites will open up precapillary sphincters, allowing the already established big flow to be distributed over the entire capillary network.

Though the distribution and the functional significance of the sympathetic cholinergic vasodilator nerves have been much studied during recent years, there are still many details which are poorly understood. The fact that they are centrally controlled from the cortex and hypothalamus, but not engaged in reflex blood pressure control, further that they are only distributed to the vessels of the skeletal muscles, and, possibly also to the coronary vessels, in itself suggests a functional engagement, under circumstances where centrally induced, prompt increases of blood supply to the muscles are needed. However the functional significance of these centrally induced reaction patterns is so far not definitely clear, though recent data make it highly probable that the sympathetic dilator fibres play an important role in the so-called defense reaction (ABRAHAMS, HILTON and ZBROZYNA 1960). — On the other hand recent experiments (HYMAN *et al.* 1959, ROSELL and UVNÄS 1960) have been interpreted as indicating that the sympathetic vasodilator fibres should open up non-nutritional vessels or 'shunts' so that the increased flow should in fact bypass the muscle capillaries and hence not serve the metabolism of the muscles directly. This interpretation led to the suggestion (UVNÄS 1960) that the dilator fibres might be activated in circumstances where the animal rather has to conserve its oxygen resources. If so, their functional significance should be the very opposite of their proposed engagement in the defense reaction, insofar as in the latter case it is implied that the dilator fibres are activated with the ultimate purpose of increasing the nutritional blood supply of the muscles.

As in this department a technique has been developed (MELLANDER 1960) which under certain circumstances allows a quantitative and selective recording of neurogenic and hormonal effects on both the pre- and postcapillary resistance vessels and the capacitance vessels, it was thought of interest to study whether the sympathetic vasodilator fibres are distributed to all the mentioned vascular sections or only to some of them, and then also to which extent this section or sections, can be influenced by the dilator fibres. Such data might shed further light on the problem of the functional significance of the sympathetic vasodilator fibres. The present results have previously been briefly outlined (FOLKOW 1959).

Method

The experiments have been performed on cats anesthetized with chloralose and urethane in amounts not exceeding 50 mg and 100 mg per kg of bodyweight, respectively. — With regard to measurement of changes in blood flow and blood volume a method recently described in a paper by MELLANDER (1960) was used. This paper should be consulted for details concerning technique and analysis of the recordings. In principle, the hind parts of the cat were completely isolated from the upper parts of the animal at the level of the hips, with exception for the aorta, the inferior caval vein and the abdominal sympathetic nerve trunks, containing the great majority

of the vasomotor fibres running to the isolated hind parts. After removal of the intestine, the inferior mesenteric artery was cannulated to measure the arterial inflow pressure of the hind parts. — The hind parts were enclosed in a water-filled temperature-regulated plethysmograph, by way of which the phasic shifts in tissue volume, and hence blood volume, could be continuously and quantitatively recorded. As in these experiments the interest was concentrated on blood flow in the skeletal muscles, the tail and the hind paws were excluded from the circulation by tight ligatures at the ankles and the proximal end of the tail. This left the blood supply intact to skin volume comprising about 5—8 % of the total enclosed tissue volume. In a few experiments the cutaneous circulation was almost entirely excluded by skinning the hind limbs, with exception of a narrow skin strip surrounding the tissues at the hip level, which was used to seal the plethysmograph. The plethysmograph was in these cases filled with Tyrode solution at a temperature of 35° C, instead of water. — In order to measure the blood flow of the hind parts, a modified Gaddum recorder was inserted in the inferior caval vein, which in this type of preparation forms the sole outflow pathway. Further, the height at which the Gaddum recorder was mounted above the animal set the venous outflow pressure level, and this level could easily be adjusted by raising or lowering the recorder. Also, the arterial inflow pressure of the hind parts could, if desired, be kept constant during an experimental procedure, by adjustment of a screw clamp, applied around the aorta proximally to the site of pressure recording in the inferior mesenteric artery. — In this way phasic changes of blood flow, blood volume, and, in certain circumstances, also of net transcapillary fluid exchange, induced, for example, by vasomotor nerve activations or injections of vasoactive drugs, could be continuously recorded, while at the same time the pressure drop across the vascular bed could be kept essentially constant. In order to study the influence of the sympathetic cholinergic dilator fibres, as compared with that of intra-arterially applied acetylcholine on the different vascular sections of the hind parts, two different procedures were used.

1. In the first series the abdominal sympathetic trunks were centrally cut and cautiously isolated in order to allow direct stimulations of their peripheral ends approximately at the height of the border between the fourth and fifth lumbar vertebra. In ten of these experiments the sympathetic vasoconstrictor fibres were acutely blocked by dihydroergotamine, given as an intra-arterial infusion into the hind parts. In five experiments the animals had been chronically depleted of catechol amines by treating them with reserpine (Serpasil ®) about mg/kg, for 4 days before the actual experiment. Thus, direct stimulations of the abdominal sympathetic trunks in this series of experiments caused an activation of the sympathetic dilator fibres, the vascular effect of which was fairly undisturbed by any concomitant excitation of the adrenergic vasoconstrictor fibres. The stimuli were delivered by a Grass stimulator at frequencies varying between 1—20/sec, a voltage of 4—8 and a pulse duration of 5 msec. — Close arterial injections or infusions of acetylcholine and other drugs could be performed via the cannulated central stump of one of the lumbar arteries.

2. In the second series (10 exp.) attempts were made to activate the sympathetic dilator fibres from their diencephalic integration centre in the anterior parts of the hypothalamus. For this purpose the head of the animal was mounted in a Horsley-Clarke apparatus, allowing a fairly exact localization of bilateral, concentric stimulation electrodes. Square-wave stimuli at a voltage of 2—5, a pulse duration of 2—5 msec and a frequency between 30—90/sec, were delivered from a stimulator Grass model S 4 C. In the course of these experiments the sympathetic dilator fibres of the hind parts were blocked by atropine and in a few experiments an essentially regional block of the constrictor fibres was induced by a slow intra-arterial infusion of dihydroergotamine into the hind parts.

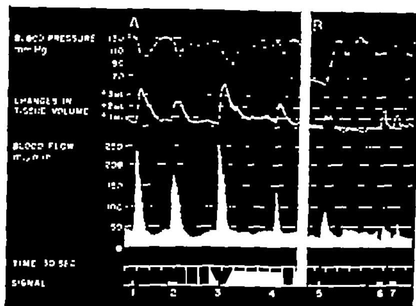


Fig 1 Serpasil-treated cat. 3.5 kg. Chloralose-urethane.
A. Vascular response in the hind quarters of
i. a. injection of acetylcholine (0.5 μg) (1)
stimulation of lumbar symp. vasodilator nerves (20 imp/sec) (2)
i. a. injection of acetylcholine (2 μg) (3)
i. a. injection of acetylcholine (0.01 μg) (4)
B. Effects on blood flow and blood volume in the hind quarters induced by
rise of arterial pressure 70 mm Hg (5)
rise of venous outflow pressure 6 mm Hg (6)
rise of venous pressure 3 mm Hg (7) (for further description see text)

Results

1 *Effects of stimulation of the abdominal sympathetic trunks* In these experiments the vasoconstrictor fibres had, as has been mentioned, either been blocked by dihydroergotamine in the course of the experiment, or by reserpine given to the animals during the preceding four days. Both types of experiments gave essentially similar results, but the constrictor fibre block was throughout more complete after reserpine treatment. This was made obvious by the fact that even maximal sympathetic excitation did not induce any vasoconstriction whatsoever in most of these experiments, when the dilator fibres had been blocked by atropine. After dihydroergotamine on the other hand, a somewhat delayed, moderate vasoconstrictor response was revealed under such circumstances, indicating that the vasoconstrictor fibres were not completely blocked by this drug. For such reasons the experiments on the reserpine-treated animals were in some respects best suited for a detailed analysis of the distribution of the dilator fibres to the different consecutive vascular sections. Further they provided the most clear-cut data concerning the extent of the dilator fibre

effect on the muscle blood vessels, as compared to the maximal vasodilator response which could be obtained by intra-arterial injections of huge doses of vasodilator agents. Acetylcholine was used here, because, under other circumstances, we have observed that the vasodilatation induced by massive muscular work is of the same magnitude as that obtained by big doses of acetylcholine and, furthermore, such a maximal exercise hyperaemia cannot be further increased by addition of acetylcholine or other blood-borne vasodilator agents. Such observations indicate that acetylcholine can be used for inducing a complete inhibition of all those vascular smooth muscles, whose tone adds to the flow resistance.

Fig 1 illustrates part of a typical experiment which from a technical point of view was entirely satisfactory and performed on a reserpine treated animal. Generally the arterial blood pressure in such animals is quite low which to a great extent appears to be due to the fact that the nervous adrenergic control of the venous side and the heart is eliminated by the drug However if only the filling pressure of the heart was somewhat raised which could easily be done by adjusting the height of the inflow from the funnel of the flow recorder it was possible to maintain a fairly 'normal blood pressure, in spite of the pharmacological adrenergic 'sympathectomy' As also the vagal nerves had been cut in the neck region, reflex blood pressure control was thus eliminated, and therefore the inflow rate from the funnel to the heart had to be very exactly balanced, if the blood pressure was to be kept fairly constant. Even minor shifts of the filling pressure of the heart could induce dramatic pressure changes, presumably by changing stroke volume and hence cardiac output, a circumstance which was utilized when the experimental procedure required sudden changes in the arterial inflow pressure. — The 'resting' blood flow was of the order of 6—8 ml/min/100 ml of tissue, and could be increased about five times by supramaximal amounts of intra-arterially injected acetylcholine, i.e. to about 35 ml/min/100 ml of tissue. Thus, if the blood pressure was kept within the normal range, 'basal vascular tone — the extent of tone left after elimination of the vasoconstrictor fibres — was essentially normal within the skeletal muscles of the reserpine-treated animals, in spite of the fact that they were completely depleted of catechol amines. It was further observed that the resistance vessels exhibited an often very striking autoregulation which incidently is clear from the relation between pressure and flow in 3 of Fig 1 B., a finding which will be dealt with in more detail in another publication (FOLKOW and ÖBERG 1961) — The biggest increases of blood flow obtained by maximal excitation of the sympathetic vasodilator fibres at the highest physiological rates, 10—20 impulses/sec, were slightly above 25 ml/min/100 ml of tissue the sympathetic stimulation illustrated in Fig 1 A, 2 resulted in a peak flow of about 25 ml/min/100 ml of tissue, but in the preceding stimulations in the same experiment somewhat higher flow values were reached.

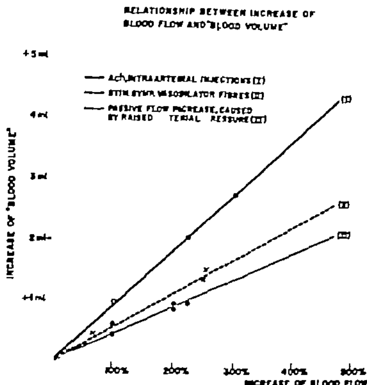


Fig. 2. Relationship between increase of blood flow and blood volume in the hind quarters of 3 kg cat as induced by i. a. injections of acetylcholine (I) stimulations of the lumbar symp. vasodilator fibres (II) and by rises of arterial perfusion pressure (III). The data are obtained from the experimen part of which is shown in Fig. 1

The maximal flow increases that could be induced by the vasodilator nerves were thus only about 20—25 smaller than those obtained by supramaximal amounts of acetylcholine. It should firstly be realized, that the studied hind parts also contain some skin and bone tissue roughly 5 and 10 volume per cent, respectively and the blood flow of these tissues, was, of course, ncluded in the flow measurement. The vasodilator fibres are known to be distributed only to the skeletal muscles but the intra arterially injected acetylcholine will reach all the tissues ncluded in the studied hind parts. Secondly for technical reasons it can hardly be expected that sympathetic stimulation at the level of the fourth lumbar ertebral disc will activate all the vasodilator fibres of the skeletal muscles the blood flow and volume of which are included n the measurement. Th se dilator fibres which are distributed to the vessels of skeletal muscles ar und the hip level will presumably have left the sympa thetic trunk at a m cranial level, a the innervation of some of these muscles originates from mor cranial parts of the spinal medulla. Furthermore some of these fibres run t rısk of being damaged when the animal is divided"

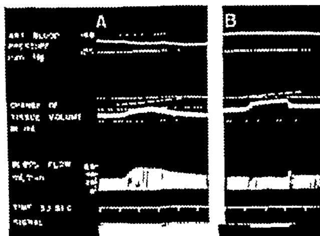


Fig. 3. Serpasile-treated cat, 3.1 kg. Chloralose-urethane. *A*. Stimulation of sympathetic vasodilator fibres successively increasing frequencies (3—6 imp/sec) so as to induce sustained vasodilatation. Note that after the slight initial increase of tissue volume, there is slower and continuous increment (indicated by adjacent dashed line) suggesting phase of outward filtration of fluid. *B*. Typical 'filtration slope' in the volume curve induced by raising venous outflow pressure 5 mm Hg.

by mass ligatures at the hip level. Thirdly any damage inflicted on the abdominal sympathetic trunks when prepared for stimulation, will, of course, tend to diminish the extent of the vasodilatation obtained on their stimulation. Thus, a number of factors, impossible to avoid completely in experiments of this type, will tend to diminish the extent of blood flow increase caused by maximal dilator fibre excitation, as compared to that obtained by supramaximal amounts of blood-borne vasodilator agents. Such circumstances make it probable that the blood flow increase in the skeletal muscles, which can be induced by intense sympathetic vasodilator fibre activation, is, — if anything, — even closer to the maximally obtainable muscle blood flow values in the intact animal than would be expected from the actual measurements in Fig. 1 and the diagram of Fig. 2. These questions will be further discussed below when the functional significance of these nerve fibres will be briefly considered. — Incidently it was a striking and constant phenomenon that, whatever frequency and stimulation characteristics were used to activate the vasodilator fibres, it was not possible to maintain an increased blood flow for more than half a minute or so if the stimulation frequency was not continuously raised throughout the stimulation period. It was, in fact, necessary to increase successively the stimulation frequency as the experiment illustrated in Fig. 3 as the event to be studied here made it necessary to try to maintain the flow increase at a steady level for some period of time. Even in such circumstances, however, a steadily increased flow could never be maintained for more than about a minute, which is in striking contrast to the longlasting, fairly steady vascular effect that can be induced by a continuous constrictor fibre stimulation. The background of this difference between the two types of sympathetic vasomotor fibres is obscure. It may be that the vasodilator fibres are more easily damaged by the artificial stimulation, though this can hardly

be the sole explanation. Another possibility is that, in some way or other they may be functionally adapted mainly for inducing phasic decreases of vascular tone, while the constrictor fibres must, of course, be fitted for their tonic vascular control. Whatever the case, this phenomenon appears to make it almost impossible to analyse such events in the neurogenically induced dilatation that require a steady state over any length of time.

It should also be observed in Fig 1 that the increase in tissue volume appears to be relatively b gger for a given flow increase when acetylcholine is injected than when the dilator fibres are activated (cf for example, 2 and 4) In the dihydroergotamine-treated animals, where constrictor fibre block is not complete, it was often observed that a flow increase was combined with almost no increase in volume on sympathetic stimulation sometimes even a decrease on volume was obtained. Acetylcholine always increased blood flow and tissue volume markedly

The diagram of Fig 2 is based on all the effects recorded in the experiment, part of which is illustrated in Fig 1 and shows the relationship between flow increase and volume increase, as caused by intra arterially injected acetylcholine (curve I) by sympathetic vasodilator fibre activation (curve II) and simply by raising the arterial pressure level (curve III) It can be seen from this diagram, which is representative of all the technically successful experiments, that — for each level of blood flow increase — the increase of regional blood volume appears to be almost twice as big when acetylcholine is distributed via the blood stream, as when the same agent is locally liberated as the transmitter at th cholinergic vasodilator nerve endings. No doubt, however the dilator fibres also cause some increase in regional blood volume if they are selectively activated, but it must be remembered that this is inevitable, even if the fibre distribution should be essentially restricted, say to the precapillary resistance vessels, and this for two reasons. From earlier studies it is known that the hind parts of a medium-sized cat contain roughly some 20 ml of blood (MELLANDER 1960) It can be approximately calculated that some 3—5 of this blood is contained within the true arterioles (see GREEN 1950 Table 1) which means that these vessels of the hind parts at normal' vascular tone contain at most 1 ml of blood. If a select ve widening of only these essels took place, big enough to increase blood flow four times, this would — according to Poiseuille s law — imply a 40 increase in internal radius and a doubling of their blood content in the present case raising the total blood volume of the hind parts by roughly 1 ml. Such an approximate deduction makes it clear that in a way the smallest arteries also form a part of the functionally defined capacitance vessels the most important section of which is constituted by the eins. — Furthermore, even if the dilator fibres cause a relaxation only of the precapillary part of the resistance vessels, this dilatation must so change the profile of the pressure drop curve along the vascular tree as to raise the distending pressure in the capillaries and the

distensible veins. Thus, other factors being constant, any dilatation of the pre capillary resistance vessels must increase the venous blood content, by way of passive-elastic distension even if their smooth muscle tone is completely un affected by the sympathetic vasodilator fibres. — Curve III in the diagram of Fig 2 illustrates the effect on blood flow and regional blood volume as induced simply by increasing the arterial pressure without and primary change in vascular smooth muscle tone. It can be seen from this curve that the passive elastic volume increase for a given increase in blood flow is very nearly the same as that caused by sympathetic vasodilator fibre stimulation. This finding is also illustrated by 5 in Fig. 1 B. '6' and 7' in Fig 1 B illustrate in another way how even slight increases in mean pressure within the venous section will definitely affect its contained blood volume. On the basis of haemodynamic considerations it can be deduced that — for a given increase of flow — mean venous pressure should be increased to roughly the same extent, whether the flow increase is due to arteriolar dilatation or to raised arterial pressure. Provided that no changes of venous tone take place, venous distension can therefore be expected to be fairly similar in the two cases. The slight difference between curves II and III is, in fact to be expected because — as mentioned above — the relaxation of the resistance vessels upon dilator fibre activation means an additional, small blood volume increase besides that due to venous distension. On the other hand, if venous smooth muscle tone had also been inhibited by vasodilator fibre activation, the volume increase could be expected to equal that caused by acetylcholine injections, where presumably all vascular sections are reached by the dilator agent. Thus these findings strongly suggest that the most important section of the capacitance vessels — the veins — is supplied with only few if any vasodilator fibres. It should then be remembered that the veins at the same time constitute the postcapillary section of the resistance vessels. — It can also be seen from Fig 1 and 2 that the volume increases in absolute values are rather small, compared with the contained blood volume in the hind parts. The reason for this is that venous tone, when deprived of its constrictor fibre control, is fairly low compared with the obviously rather marked 'basal tone' of the resistance vessels. The extent of volume increase caused by acetylcholine injection was far bigger if the initial tone of the capacitance vessels had been raised by an intra-arterial infusion of for example, noradrenaline.

Fig 3 illustrates in another way the fact that vasodilator fibre activation evidently causes an increase of intravascular pressure at and beyond the capillary level, which should also be the case if these fibres are predominantly or solely distributed to the precapillary section of the resistance vessels. In this experiment a moderate, but steadily maintained blood flow increase was induced by dilator fibre stimulation, which, as mentioned earlier made it necessary to increase the stimulation frequency continuously The onset of stimulation is accompanied by an initial, rather rapid phase of flow and volume

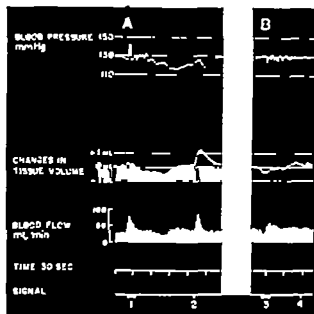


Fig 4 Cat 3.2 kg Chloralose-urethane

A. Vascular responses in skeletal muscles of the hind quarters of the cat, evoked by topical hypothalamic stimulation activating the cholinergic vasodilator nerves (1) and by close arterial injection of 0.01 μg acetylcholine (2). Although blood flow is about equally increased in both cases, the regional tissue volume (blood volume) is increased by acetylcholine but hardly affected during hypothalamic stimulation

B The same experimental steps as in *A* performed after atropinization. The vasodilator responses are now abolished. A slight vasoconstriction is obtained by hypothalamic stimulation (3)

increase, in time well co-ordinated with each other Once blood flow is stabilized at its higher level however it is accompanied by a slow but continuous increase of tissue volume (A in Fig 3) which in character is very similar to that induced by a slight rise in venous pressure (B in Fig 3) In the analysis of the experimental technique used, MELLANDER (1960) presented strong evidence to show that such a secondary continuous volume increase is due to an outward filtration caused by raised capillary pressure. As here arterial inflow pressure and venous outflow pressure are kept constant, this effect of dilator fibre stimulation suggests that the main resistance decrease is located proximally to the capillary level. Owing to the consequent shift in profile of the pressure drop along the vascular bed, it can be expected that venous pressure must also be somewhat raised leading to a passive venous distension. Always, when it proved technically possible to maintain a steady level of increased flow by dilator fibre stimulation long enough to perform such a measurement, there was evidence of outward filtration If a steady flow level cannot be maintained the present technique does not allow an exact evaluation of the fraction of the volume shift that is due to transcapillary filtration exchange It can be expected however that the extent of outward filtration is much more marked at for instance, maximal activation of the dilator fibres, but as soon as huge flow increases are induced, they are as mentioned always very transient.

2. *Effects of hypothalamic stimulation.* In these experiments the sympathetic vasodilator fibres were activated by topical stimulation of their diencephalic integration centre The effect of a hypothalamic stimulation on regional muscle

blood flow and blood volume is illustrated in Fig 4 1 where the activation of the dilator fibres causes a blood flow increase of about 150 % To eliminate the passive changes of blood flow and blood volume, due to blood pressure changes in connection with the stimulation, the arterial inflow pressure was kept fairly constant by adjusting a screw clamp placed around the abdominal aorta. — It can be seen from the figure that the prompt blood flow increase is combined with very little shift in tissue volume, followed by a somewhat delayed phase of volume decrease. On the other hand, injection of acetylcholine (Fig 4 2) in a concentration that gives about the same blood flow increase, causes a clear-cut increase also in volume. Atropine eliminates the acetylcholine effect and the *increase of flow on hypothalamic stimulation*, leaving a slight decrease in flow and volume as the only regional vascular effects consequent to the stimulation. — The effect of hypothalamic stimulation on the tissue volume varied to some extent in some experiments there was an actual decrease in volume at the peak of the flow increase, in others the volume increased slightly but always to a lesser extent as compared with the effect of injected acetylcholine in amounts that elicited an equal blood flow increase. — It was mentioned in the previous section that the dilator fibres appear to be distributed essentially to the precapillary resistance vessels, but that their activation nevertheless must lead to a certain volume increase, due to a passive distension of the venous side It does therefore, in fact, appear that the topical hypothalamic stimulation, — beside the activation of the vasodilator fibres — also to some extent activates vasoconstrictor fibres to the capacitance vessels of the muscles. Otherwise it can hardly be explained how sometimes blood flow was increased by hypothalamic stimulation, while there was no change or even an actual decrease in tissue volume.

Thus, the results of the hypothalamic excitation of the sympathetic vasodilator fibres also suggest that they are essentially distributed only to the resistance section of the vascular bed in skeletal muscles. Furthermore, it appears that the cardiovascular reaction pattern, which is induced from their diencephalic centre, may to some extent also involve activation of the vasoconstrictor fibres of the capacitance section of the vascular bed *in* the muscles.

Discussion

The present experiments indicate that, under favourable experimental conditions, the sympathetic vasodilator fibres, when activated at the highest physiological rates, are capable of inducing blood flow increases in the skeletal muscles which in extent are close to the state of maximal dilatation. As, further a number of unavoidable experimental artifacts, — unlikely to affect the estimation of maximal vasodilatation — tend to diminish the neurogenic vasodilator response, it is in fact probable that the present results if anything underestimate the potency of the vasodilator fibres.

The question then arises whether the sympathetic vasodilator fibres really affect the same set of vessels within the muscles as the metabolites, which are locally released in muscular work. As briefly mentioned in the introduction, a series of studies of the dilator fibre effect on the relationship between volume flow and diffusion exchange have led to the suggestion that the dilator fibres might open up non-nutritional shunts parallel-coupled to the capillary bed. No doubt stimulation of these fibres can in some experimental circumstances create a situation that deserves to be called 'functional shunting' For the evaluation of the physiological significance of the dilator fibres it is, however most important to distinguish between true shunt vessels and the occurrence of a functional shunting due, for example, to an uneven distribution of the capillary flow For such reasons this problem will here be dealt with in some detail evaluating the present results against the background of a number of well-established data on muscle blood flow and oxygen consumption.

If for a moment, it is presumed that true shunt vessels, controlled by the cholinergic vasodilator fibres, exist, acetylcholine can then hardly be imagined to lack the power of relaxing these shunt vessels when distributed *via* the blood stream in high concentrations. The injected acetylcholine must, by way of the capillary walls, promptly reach the tissue fluid which form the immediate environment of all cells, including the presumed shunts and their cholinergically innervated smooth muscles. To draw a parallel, the adrenergically innervated shunts in the skin certainly respond sensitively to catechol amines in the blood For such reasons it has to be assumed that the maximal flow value obtained by supramaximal amounts of intra-arterially injected acetylcholine must also include the blood flow increase through the shunts, if they really exist. — Further acetylcholine and related substances are known to dilate the nutritional blood vessels of the muscles powerfully being in fact able to induce the same extent of maximal flow as can be elicited by intense muscle activity Acetylcholine, intra-arterially injected at the peak of a maximal work hyperaemia, is in our experience not able further to enhance the blood flow increase Now if it is tentatively assumed that specific muscle shunts exist, such findings must mean either that both the nutritional vessels and the shunts are dilated by the locally released metabolites or that the shunts are unaffected both by the metabolites and by injected acetylcholine. As mentioned above the latter alternative is highly unlikely and therefore it is necessary to consider the possibility that muscular work also causes a dilatation of the presumed shunts.

When this question is dealt with, it must be remembered that the vasodilator fibres can induce almost the same extent of flow increase as supramaximal concentrations of acetylcholine or intense muscular work. To take some actual figures from the experiment, illustrated in Fig 1 and 2 maximal dilatation here implied a blood flow of 35 ml min 100 ml of tissue while intense dilator fibre activation increased flow slightly above 25 ml min

100 ml of tissue. The 'resting' blood flow in the studied tissue part, consisting of up to about 85 % skeletal muscle, is in our experience around 3—4 ml/min/100 ml of tissue, under circumstances where a normal resting constrictor fibre tone is present, and about twice as high when the vessels are sympathectomized. If true shunt vessels really exist, it then follows that the margin left for increasing the nutritional blood flow of the muscles would be at best some 300 % in the intact animal, i. e. it would be constituted by the difference between the flow figures of 35 ml and 25 ml. As mentioned earlier this difference may in reality be even smaller as with the present technique the extent of the dilator fibre influence is — if anything — underestimated. One is thus forced to assume that the greater fraction of the increased cardiac output in muscular work in fact bypasses the muscle capillaries, if true shunts exist, being of no use for the nutrition of the muscles but only adding to the load on the heart.

Already the above-mentioned considerations thus make it clear that the existence of any significant extent of specific shunts within the skeletal muscles is highly unlikely In addition, the above mentioned flow figures, — if tentatively interpreted in terms of a dual shunt and 'nutritional' muscle circulation, — are incompatible with known data for the relation between maximal oxygen uptake in intensely working muscles and their maximal blood flow If we here exemplify with data taken from man, a resting subject can be considered to consume about 250 ml oxygen/min, where roughly 20 % can be calculated to be consumed by the skeletal muscles, at a muscle blood flow of about 2—4 ml/min/100 ml of tissue. The blood flow can at best be increased some 15—20 times, to about 40—50 ml/min, while during heavy muscular work the oxygen extraction per unit blood volume can increase roughly 3 times. During intense muscle activity the total oxygen consumption of the muscles can increase well above 50 times, as the maximal oxygen uptake in man then often reaches 3,500—4 000 ml/min, most of which is taken up by the muscles. It is mathematically impossible to account for this huge increase of oyygen uptake by the working muscles, if any significant fraction of their maximal blood flow bypassed the capillaries. Further the venous blood coming from intensely working muscles can be almost depleted of oxygen, which would not have been the case if a bigger fraction of it had passed shunts. Data concerning maximal oxygen uptake, etc. in cats are not available, but it is hardly likely that they would be profoundly different from those in man, when corrected for the difference in body size. Thus, there appears to be no room left for any specific shunts in the muscle circulation, if it is not assumed firstly that they are normally closed in a state of intense tonic contraction and only opened by activation of the dilator fibres, secondly that their cholinergically innervated smooth muscles are entirely unaffected even by massive concentrations of acetylcholine and vasodilator metabolites in their immediate environment. Such assumptions are so untenable that it seems necessary to reject the theory

that any significant number of specific shunts exist in the muscles. Other ways must then be found to interpret the series of data which undoubtedly suggest the establishment of a 'functional shunting' when the dilator fibres are experimentally activated (HYMAN *et al.* 1959, ROSELL and UVNÄS 1960).

The present study indicates that the vasodilator fibres are mainly distributed to the precapillary resistance vessels, because, — when related to the same flow increase induced by injected acetylcholine, — their activation causes a much smaller increase of regional blood volume, not bigger than can be predicted to be the result of a selective arteriolar dilatation and the consequent passive-elastic distension of the venous side. It appears therefore that the venous side, constituting the postcapillary resistance vessels and also the main part of the capacitance vessels, is not directly influenced by the dilator fibres. Also, the fact that the dilator fibres appear to bring about a raised mean capillary pressure is in harmony with such a selectivity in fibre distribution. — Suppose now that the dilator fibres only make contact with more proximal sections of the precapillary resistance vessels, i. e. the true arterioles, but not with the smallest arterial ramifications, which can be called the 'precapillary sphincter sections' *controlling the number of capillaries open to flow*. A selective, intense activation of the dilator fibres will then bring about a markedly increased flow — but not a maximal one, as not all vascular sections contributing to the flow resistance are widened. This increased flow will, however, pass essentially the same, limited fraction of the capillary bed that is normally open in the resting muscle. It can even be expected that some of the capillaries may become closed, as in electrical stimulations it is unavoidable to induce some vasoconstrictor fibre effects also, except in animals severely treated with reserpine, and the constrictor fibres also affect the precapillary sphincter sections to some extent (see e. g. FOLKOW 1955, FOLKOW and MELLANDER 1960 and ROSELL and UVNÄS 1960). — Furthermore, it is well known that the smooth muscles of the vascular sphincter regions relax and open up previously closed capillaries when tissue metabolism is increased. They may react in a similar way when, for instance, blood flow is artificially reduced, other things being constant. Hence, it is not unlikely that they react in the opposite direction, i. e. constrict somewhat, when an increased blood supply is suddenly forced upon them by an upstream, strictly localized dilatation, because of the consequent changes in their immediate chemical environment and the regional increase in transmural pressure (FOLKOW and ÖBERG 1961).

Thus, an experimental situation may here be created, where an increased volume flow passes through an unchanged, or even somewhat rarified capillary network. As has been previously briefly discussed (FOLKOW 1960), such a situation implies firstly a reduced time for diffusion exchange in the individual capillary, secondly an unchanged or even a somewhat reduced capillary surface available for this exchange and thirdly a slightly increased mean diffusion distance from the blood to the tissue cells. This means that limited

sections of a skeletal muscle are provided with a great excess of nutritional supply while other parts of the muscle get little or nothing. In other words, a 'functional shunting' is experimentally created, where no specific shunts or any new concepts of vascular smooth muscle behaviour are implied only a distribution of the dilator fibres restricted to the bigger precapillary resistance vessels. Such a view is in harmony with recent studies on capillary permeability in skeletal muscles (RENKIN 1959 a, b) which suggest that shunting of blood in skeletal muscles is merely a matter of a variable distribution of flow within the skeletal muscles, while no evidence of specific shunt vessels could be found. It appears in fact that available data on the characteristics of the dilator fibre effects, also those of HYMAN *et al.* (1959) and ROSELL and UVNÄS (1960) do not necessitate the implication of true shunt vessels, but can be explained along the above mentioned hypothesis of a selective dilator fibre distribution. In quite recent experiments, utilizing a different experimental approach, RENKIN and ROSELL (1961) have arrived at a similar view. It is haemodynamically important to distinguish such a point of view as there is a great difference between a vascular bed containing both nutritional vessels and parallel-coupled shunts and one with only the former type of vessels, where occasionally a type of 'functional shunting' can occur.

If this is so the centrally induced visceromotor pattern, of which the dilator fibre activation forms a dominant part (see UVNÄS 1960) is excellently fitted for participation in alarm reactions, as ABRAHAMS, HILTON and ZBROZYNA (1960) suggested. The immediate, dramatic redistribution of an increased cardiac output, favouring the skeletal muscles, implies an anticipation of the nutritional demands, arising at the moment the skeletal muscles are brought into action in a flight or defence reaction. The main pattern of blood flow is then already centrally induced, and as soon as the muscles are activated, the locally released vasodilator metabolites only have to open up all the capillaries, to spread out the huge blood flow over the entire capillary surface of the muscles.

The present hypothesis of a restricted vasodilator fibre distribution to the bigger precapillary resistance vessels only further implies that the increased flow can be established with a minimum of blood 'pooling' within the muscles, as few if any dilator fibres appear to run to the venous side. It may even be so that the blood volume contained in the muscles can decrease in situations when the dilator fibres are centrally activated in the intact organism. This would be possible if the constrictor fibres to the venous side of the muscle vascular bed were concomitantly activated to some extent, as appeared to be the case at least in some of the present topical stimulations of the hypothalamus.

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From the Scripps Institution of Oceanography, University of California, La Jolla, California, U. S. A.

Physiological Adjustments to Prolonged Diving in the American Alligator

Alligator mississippiensis

By

HARALD T. ANDERSEN[1]

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Abstract

ANDERSEN, H. T. *Physiological adjustments to prolonged diving in the American alligator. Alligator mississippiensis.* Acta physiol. scand. 1961. 53. 23—45. — Conspicuous physiological adjustments to experimental submersion have been observed in the natural divers among the birds and the mammals. In order to obtain further information on the phenomena associated with water immersion, and to explore these characteristics in other diving vertebrates, experiments have been carried out on the American alligator, *Alligator mississippiensis.* The heart rate was found to slow down during diving. The pressure induced in the large arteries by ventricular systole diminished only gradually during diastole probably due to an increased peripheral resistance resulting from a marked vasoconstriction in certain vascular beds. This thesis was supported by the fact that lactic acid formed in the muscles during diving did not appear in the blood until after emersion, suggesting that the blood flow through the muscles is greatly reduced while the animals dive. The alligator may recover easily from a submergence lasting until the oxygen stores are almost completely depleted. These results indicate that the "diving reflexes" occur in a wide variety of vertebrate divers belonging to different phylogenetic groups. They also support the following conclusions:

1) The circulatory adjustments, by shutting off the perfusion to certain vascular beds, enable the alligator to make the limited oxygen stores last throughout prolonged periods of submersion.

[1] Present address: Department of Physiology, Kungl. Veterinärhögskolan, Stockholm 51, Sweden.

2) The animal remains in a state of useful consciousness until the oxygen stores are almost exhausted. This serves to extend the safety margin for prolonged diving and ensure a complete utilization of the oxygen available.

Certain animals among the vertebrates exhibit a high degree of adaptation to aquatic life, although they are equipped with essentially the same respiratory and circulatory organs as terrestrial forms. Some of these animals are known to be able to remain under water for an extended period of time and endure a very high degree of asphyxiation. While submerged, they may be observed at rest, as well as carrying out various activities, such as feeding, cruising or catching prey.

The physiological adjustments which permit the diving mammals and birds to endure prolonged periods of submersion have been studied by several investigators. Since BERT (1870) started these studies, important contributions have been made by RICHET (1899), LOMBROSO (1913), IRVING *et al.* (1941 a, 1942), SCHOLANDER (1940) and SCHOLANDER *et al.* (1942). The subject was reviewed by IRVING (1939).

The observations of these investigators have indicated certain factors to be particularly important for prolonged diving:

1) Large oxygen depots, and selective and complete use of these stores.
2) Relative insensitivity to effects of asphyxiation on respiration.
3) Reduced metabolism during diving.

A fair amount of knowledge of the physiological adjustments to prolonged diving in birds and mammals has been established, whereas similar information on the poikilothermic divers is scarce. There are diving animals both among the amphibians and the reptiles which presumably would be well suited for experimental work. The amphibians are known to remain under water for extremely long periods of time, but in these animals the cutaneous respiration is extraordinarily large, and it may well be that the cutaneous component of respiration is adequate to maintain life during diving. The reptiles are, however, almost entirely dependent on their lungs for respiratory gas exchange. It is, thus, very desirable to study the diving reptiles in order to make our knowledge of the physiological function in the natural divers more complete from a comparative point of view.

Among the reptiles, the crocodilians are well suited for such a study. The crocodilians are semiaquatic. They never depart far from water, and when disturbed they will try to escape into the water and submerge themselves. Their fitness in an aquatic habitat is also evident from their hunting. They catch their prey in water, or they pull the victim into the water and submerge him until drowning.

A few publications deal with the diving ability of the reptiles, but only to a very limited extent (DILL and EDWARDS 1931, JOHANSEN 1959, WILBER 1960). The present investigation was carried out on the American alligator

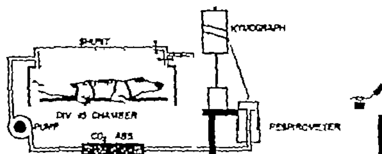


Fig. 1 Experimental design for the study of metabolism by indirect calorimetry

Alligator mississippiensis, which, like the other crocodilians, is amphibious, and displays a striking ability to endure prolonged diving

Materials and Methods

Animals and animal care

Fourteen alligators were used in the present study which was carried out from July 1958 to September 1959. The animals were kept in the laboratory at temperatures ranging from 22 to 27° C. They were fed horsemeat and bone meal. At times some of them had to be fed forcibly. None of the animals stayed at the institute for more than 3 months at a time. The weight of the animals varied little over the period which they spent at the institute.

The alligator is known to show marked seasonal changes as to activity and feeding habits. These changes were ignored in the present investigation because seasonal changes in the ability to perform prolonged diving did not occur as far as could be judged.

Arrangement of the diving experiments

Almost all of the data presented in this study has been obtained from experiments in which the animal was involuntarily submerged. The alligator was strapped down on a weighted board like the one shown in Fig. 1 so that diving could be induced by gently tilting the board. It was noticed that the animal would display the same characteristic adjustments to diving when only the nares were under water as they did when the body was completely submerged. Advantage was taken of this phenomenon in the experiments where the blood pressure recordings were done. Thermal disturbances during the experiments were avoided by insuring that the water in which the alligator was submerged was of the same temperature as the room in which the animals were kept.

Methods employed in the studies of the circulation

The heart rate was recorded between needle electrodes inserted subcutaneously into the right forelimb and the left hindlimb. Electrocardiograms were obtained both in the involuntary dives, and in dives performed spontaneously by an animal swimming freely and diving voluntarily. The recordings were obtained by means of a Sanborn Viso-ette Electrocardiograph Model 300 or a Grass Electroencephalograph Model III D.

In order to secure information on the arterial blood pressure, the femoral artery was exposed, and a polyethylene catheter i.d. 0.6 mm was inserted approximately 5 cm into this vessel. The catheter was attached to a calibrated Statham pressure transducer Model P23AA. The pressures were recorded by a Gilson Electronics Medi-polygraph.

During the experiments dealing with measurements of blood pressures, only the nares of the animals were submerged. Diving conditions could therefore be accomplished by a minute tilting of the board, and changes in hydrostatic pressure due to this procedure could not be observed on the recording equipment. Sequences of the heart rate and the blood pressure were recorded continuously for at least 30 seconds at a time.

Methods used in the studies of respiration

Samples of arterial blood were withdrawn from the femoral artery which had been cannulated as described above. The blood samples were analyzed for their content of oxygen, carbon dioxide and lactic acid. The pH of the arterial blood was also measured. The syringes into which the blood samples were drawn contained heparin and a 10 sodium fluoride solution in the dead space. A little mercury was drawn into the syringe after the blood in order to provide a gas tight seal, and to facilitate adequate mixing of the blood before an aliquot was removed for analysis. Until the analytical work could be performed, from 2 to 8 hours after sampling, the syringes were stored in a bath of ice and water. The technic for anaerobic handling of the blood samples outlined by ROUGHTON and SCHOLANDER (1943) was followed closely.

Since the oxygen of the blood constitutes a very important part of the total quantity of oxygen available during diving the amount sampled for analytical purposes had to be kept at a minimum. This requires the exclusive use of microtechnics in the blood analysis. By means of such procedures, it was possible to keep the individual blood samples at 0.8 ml each as this amount proved sufficient for determinations in duplicate of all of the parameters referred to below except for the pH which was always measured separately. To prevent working with anemic animals usually not more than two experiments involving sampling of blood were performed on any animal.

The content of oxygen in the blood was determined by means of the Scholander Roughton Syringe Method (ROUGHTON and SCHOLANDER 1943). The reagents described in the original paper did not appear useful when applied to alligator blood. This finding was not surprising considering that the method was first developed for the blood of homoiothermic animals, and especially for mammalian blood. By developing a different set of reagents, SCHOLANDER and VAN DAM (1956) successfully analyzed blood from different species of fishes by the Syringe Method. This modified procedure also failed to work satisfactorily on alligator blood, however because the solution No. 5, the acid sulfate solution, did not break down the blood proteins so as to constitute a flocculent precipitate in the syringe. By increasing the acidity of this solution so that it was made up of 30 g Na_2SO_4 anhydrous in 100 ml of water to which 8 ml concentrated H_2SO_4 was added, it was made applicable to alligator blood. The amount of carbon dioxide in the blood was also determined in the Scholander-Roughton Syringe as described by SCHOLANDER, FLEMISTER and IRVING (1947).

Determinations of the concentration of lactic acid in the blood samples were carried out according to the method of BARKER and SUMMERSON (1941) and modified by STROM (1949).

The pH of the arterial blood was measured by means of a Beckman Zeromatic pH meter provided with Beckman 39022 Hypodermic Style Electrode Assembly which permits pH measurements on 0.5 ml of blood while the sample is drawn.

The blood volume was estimated in two of the alligators by bleeding and washing out the vascular system with oxygenated Frog Ringer's solution. Such a wash-out method is especially well applicable to poikilothermic, diving animal like the alligator because the heart will keep on beating for hours after almost all the blood has been drained, thereby ensuring an adequate wash-out. The oxygen capacity of the blood was determined on blood samples equilibrated with air and analyzed for oxygen as described above.

Gas from the lungs was sampled at suitable intervals during diving. For this purpose the trachea was cannulated with piece of plastic tubing i. d. 0.8 mm which was inserted approximately 10 cm. A sampling bulb of capacity 6 ml was ttached to the poly ethylene tubing. Gas could be withdrawn from the lungs into the sampling bulb by lowering mercury-filled levelling bulb connected to the sampling bulb. Before a sample was taken, the sampling bulb was filled with gas from the lung air the latter being subsequently returned to the animal through the catheter. This procedure was repeated two to three times, thus, ensuring a certain degree of mixing of the gas in the lungs before the actual sample was removed. Not more than 2 ml of lung air was withdrawn in each sample. This amount of gas provided an ample supply for d plicate determinations of the content of carbon dioxide and oxygen in the sample as analyzed in the 0.5 ml Analyzer (SCHOLANDER 1947).

The lung volume of two of the animals was estimated by means of total body plethysmography. The plethysmograph was made from a heavy-walled plastic tube. A manometer tube was taken from a Van Slyke Manometric Blood Gas Apparatus and built into the plethysmograph by means of a plastic connector fitted with two o-rings which sealed around the manometer tube. The plethysmograph was also supplied with small faucet into which the nozzle of a 20 ml syringe fitted very accurately. The animal was introduced into the plethysmograph which was subsequently filled with water and sealed off. The formation of air bubbles along the inside wall of the plethysmograph was avoided by coating with a very small amount of detergent.

In an experiment an initial pressure P was read off the manometer the corresponding, unknown lung volume being V_1. A small amount of water ΔV was introduced into the plethysmograph from the syringe. Hereby the lung volume diminished to value $V =$ $(V - \Delta V)$. The pressure would simultaneously increase from P to $P = (P + \Delta P)$ which could be read off the manometer. From this information V_1 is calculated as follows

$$V_1P = V_2P = (V - \Delta V)(P + \Delta P)$$

$$V_1P = V_1P + V_1\Delta P - P\,\Delta V - \Delta V\Delta P$$

$$V\,\Delta P = P_1\Delta V + \Delta V\,\Delta P = \Delta V(P_1 + \Delta P)$$

$$V = \frac{\Delta V(P + \Delta P)}{\Delta P}$$

Indirect calorimetry for the study of metabolic rate

The consumption of oxygen before and after submersion was registered continuously on the kymograph of the closed circuit pparatus shown in Fig. 1. The diving chamber was ventilated by means of pump. The carbon dioxide in the system was absorbed in a column containing Ascarite and the gas lost in this way was replaced with 100 % oxygen from the respirometer. The diving chamber was made from plexiglas so that the animal could be observed during the experiment.

Results

Performance of prolonged diving in the alligator

Most of the alligators used for th present study were 2—6 years old. When they were free to swim and dive in a large pool, they were observed to submerge 30—60 min at a time.

Ascarite (Registered Trade Mark) sodium hydroxide-asbestos mixture with CO absorbing properties.

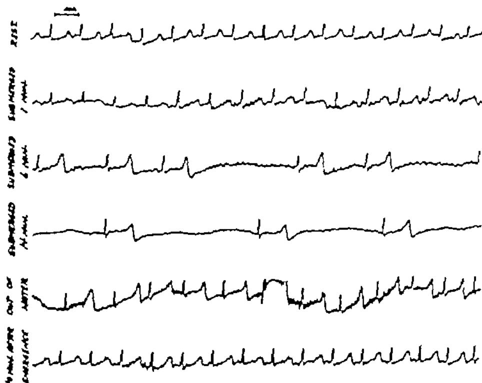


Fig. 2. ECG-tracings taken before, during and after a 15 minutes dive, showing the development of the diving bradycardia.

Heart rate and electrocardiographic changes

A very conspicuous bradycardia was observed in the diving alligator. The heart rate decreased gradually rather than instantaneously, at least during the forced dives, and the period of time required to establish a constant, low frequency varied considerably in the different individuals. The changes in the electrocardiogram associated with diving are shown in Fig. 2 as recorded between right forelimb and left hindlimb. A transient arrhythmia was always observed to take place during the period in which the diving bradycardia developed, as a ventricular contraction might be followed by a prolonged diastole. In the earlier stages of the dive this prolonged diastole appeared quite irregularly and its duration varied markedly. The corresponding electrocardiogram, therefore, consisted of groups of fairly regular heart beats separated from each other by an extended diastole. The ECG-tracings furnished in Fig. 2 show that the fully developed bradycardia is caused mainly by the prolonged diastoles. In the pre-diving period the total time required for the registration of the electrical events associated with one complete cardiac cycle was 1.30 sec.

The T wave diminished rapidly at the onset of the dive and during the

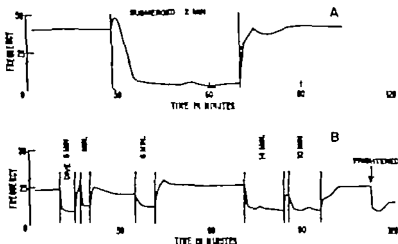


Fig. 3. A graphical representation of the diving bradycardia.
A. From an involuntary dive.
B. From series of voluntary dives.

first minute of submersion it was hardly noticeable. When it reappeared, the amplitude showed a marked increase. The T wave was frequently diphasic or completely inverted while the animal was submerged. The increased amplitude of this wave persisted usually for several minutes after emergence.

Fig. 3 A is a graphical representation of variations in the heart rate during an experiment in which the alligator endured involuntary submersion for 42 min. The heart frequency was 41 beats per minute in the pre-diving period. An initial, slight increase in the heart rate took place before the bradycardia started developing. Within 10 min the frequency of the heart had dropped to 2—3 beats per minute. The bradycardia persisted during periods of vigorous struggling in conformity with observations on the seal (SCHOLANDER 1940) and the duck (ANDERSEN 1959 a). The data shown in Fig. 3 B was obtained from an animal swimming freely in a large tub, diving voluntarily. In this case, the pre-diving heart rate was 24 beats per minute. The frequency of the heart did not drop down to such an extremely low level as the one shown in Fig. 3 A, but was maintained at 30—50 % of the pre-diving rate. However, the time required for the establishment of this degree of bradycardia was much less than in the forcibly submerged animal. The pre-diving heart rate was rapidly restored upon emersion from involuntary dives as well as from voluntary ones.

In one experiment in which the right vagus was cut in one of the alligators, no bradycardia developed during diving.

Arterial blood pressure

Experiments were conducted in which the changes in arterial blood pressure during diving was studied in the femoral artery. Sections of a record obtained

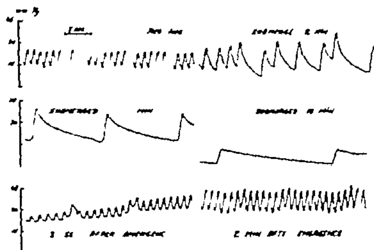


Fig. 4 Tracings showing the arterial blood pressure in the femoral artery during 20 minute di

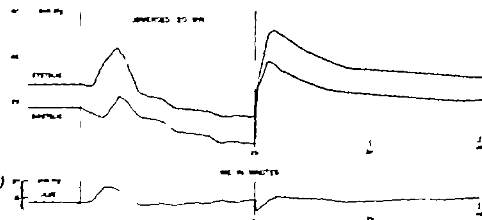


Fig 5 A graphical plot of the systolic diastolic and pulse pressures in the femoral artery of diving alligator

in one of the experiments are presented in Fig 4 The systolic dia tolic and pulse pressures fr m th same experiment were plotted graphically from the continuous record a d are given in Fig 5 The average value for these three pressures wa 28 17 and 11 mm Hg respectively in the pre-diving period. During the earl est part of the di e all three pres ures increased very markedly After this initial se t lic and diastolic pres ure fell throughout the period of submergence The dia tolic pressure approached zero toward th end of the dive. Th pulse pres ure a maintained fairly cor tant during diving except for the ir t al transient rise. Upon emersion, both the syst lic

and diastolic pressure rose steeply to a value of approximately twice the resting while the pulse pressure dropped equally abrupt to a very low level. The latter pressure was restored to th pre-diving value in about one minute, whereas the recovery of the systolic and diastolic pressure was not completed until approximately one-half hour after emersion.

During diving the pressure induced in the large arteries by the ventricular contraction decreased very slowly (Fig 4) indicating that the rate of emptying of these large blood vessels became extremely retarded.

Blood volume

Two alligators were sacrificed in order to estimate their blood volume by bleeding and washing out the vascular system with oxygenated Frog Ringer's solution. The results are given in Table I below

Table I The blood volume of the alligator

Weight	Blood Volume	Blood volume as per cent of body weight
2.7 kg	140 ml	5.1
7.5 kg	410 ml	5.5

Oxygen capacity of alligator blood

Samples of blood were withdrawn from three animals for determinations of the oxygen capacity of alligator blood. The samples were equilibrated with air and analyzed for oxygen. The values obtained are presented in Tabl II.

Table II The oxygen capacity of alligator blood in Vol. %

Date	Observed O_2-capacities			Average
8—28—58	10.2	10.7	10.1	10.5
9—10—58	10.2,	9.6		9.9
3—12—59	8.2,	7.9		8.1

The content of oxygen and carbon dioxide in the arterial blood

An illustration of the simultaneous changes in the oxygen and the carbon dioxide content of the arterial blood during a dive is presented in Fig 6 Figs. 7 and 8 give additional data on the amount of oxygen in the arterial blood in two di es of comparabl duration. The slopes of the curves are generally steeper in the initial part of the dives than towards the end, showing that oxygen is consumed more rapidly during the earlier stages of the submersions than

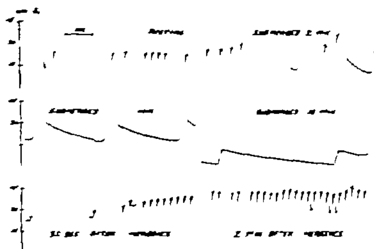


Fig. 4. Tracings showing the arterial blood pressure in the femoral artery during [illegible] dive.

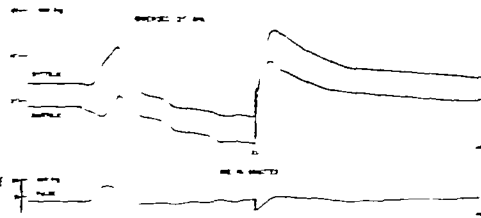


Fig. [illegible] A [illegible] the [illegible] diastolic and pulse pressures in the femoral artery of [illegible]

[illegible] of [illegible] experiments are presented in Fig. 4. The [illegible] diastolic and pulse pressures [illegible] same experiment were plotted graphically from the [illegible] in Fig. [illegible] The average [illegible] for these three pressures was [illegible] mm Hg respectively in the pre-dive period. During the [illegible] pressures increased [illegible] marked [illegible] and diastolic pressure fell [illegible] period of [illegible] The [illegible] pressure [illegible] end of the dive. The [illegible] pressure was maintained [illegible] during [illegible]

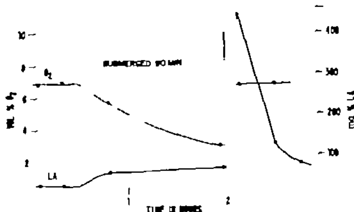


Fig. 8. Concentrations of O_2 and lactic acid in the arterial blood of an alligator before, during and after a strugglesome dive.

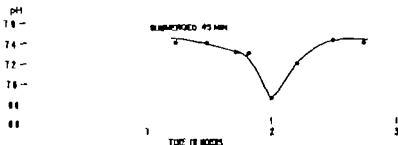


Fig. 9. Variation in pH of arterial blood before, during and after diving.

increased relatively little during submersion. On emersion, however, the blood was rapidly loaded with lactate. The dive illustrated in Fig. 7 was quietly endured, whereas in the other dive, Fig. 8, the animal exhibited bursts of vigorous activity throughout the period of submergence. This, apparently resulted in a very high level of lactic acid in the arterial blood upon ascent. Contrary to what should have been expected, it appears that the arterial blood is depleted of oxygen at a faster rate in the initial part of the quiet dive than in the other. The arterial blood also contained more oxygen at the end of the strugglesome dive than in the quiet one.

The pH of the arterial blood shows a moderate fall during the dive succeeded by a sudden and very conspicuous drop in the post-dive period (Fig. 9).

Lung volume

The lung volumes of two alligators were measured by means of total body plethysmography. The one, having a body weight of 4.4 kg, was found to have a lung volume of 450 ml. This figure was confirmed in a second experiment.



Fig. 10. Changes in the O_2 and CO_2 content of lung air during a prolonged dive.

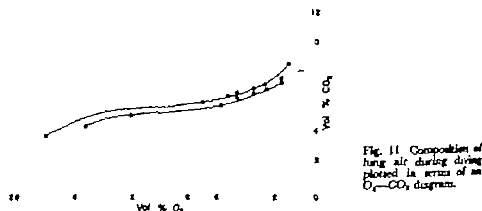


Fig. 11. Composition of lung air during diving plotted in terms of an O_2—CO_2 diagram.

The body weight of the other animal was 3.7 kg. A series of determinations of the lung volume of this animal was carried out. It was estimated to be in the range of 280 to 330 ml. It is very difficult to determine the "normal" inspiratory lung volume of a crocodilian to a fairly narrow range because it varies widely from inspiration to inspiration.

Variations in the composition of the lung air during diving and the rate of oxygen consumption from this depot

In the beginning of a dive the alligator used oxygen at a fairly rapid rate. As much as one half of the oxygen in the lung air may be consumed during the first 20 min. of a 2 hour dive. During the rest of such a dive, the oxygen of the lung air was used at a much slower rate (Fig. 10). The concentration of CO_2 in the lungs increased slowly throughout the period of submergence. It was found that the content of this gas rises more rapidly during the initial part of the dive than later. At the end of the submersions which lasted until almost all of the oxygen of the lung air was used up, a relatively steep increase in the carbon dioxide concentration was commonly observed. These patterns of the respiratory gas exchange may also be well illustrated in terms of an O_2—CO_2



Fig. 12. The depletion (dots) and consumption (columns) of oxygen from the lung air during diving.

diagram like the one of Fig. 11. Here the simultaneous concentrations of oxygen and carbon dioxide have been plotted against each other. The shape of the curves is sigmoid like in all diving animals from which comparable data have been obtained (SCHOLANDER 1940, ANDERSEN 1959 b).

The lung air is the largest oxygen depot in the alligator. The rate of consumption of oxygen from this store during diving would therefore be of great interest. It has already been shown how the concentration of oxygen in the lung air varies with diving time (Fig. 10). If the lung volume did not vary during breath holding, the actual amount of oxygen in the lungs at any time could easily be calculated from this curve. Due to the shrinkage of the lungs during diving such a direct calculation is not feasible. It is, however, still possible to quantitate the data from the gas analyses by making the assumption that the amount of nitrogen in the lung air does not change appreciably during diving. This may be expressed by the equation

$$V \cdot \% N = V'' \cdot \% N''$$

Here V is the lung volume at the start of any interval considered, and $\% N$ is the nitrogen concentration of the lungs in volumes per cent at the same time. V'' is the lung volume at the end of the interval, and $\% N''$ is the corresponding nitrogen concentration of the lungs. The values for $\% N$ can be obtained from the gas analyses by subtracting the sum of $\% CO_2$ and $\% O_2$ from 100. Using these values for $\% N$ and $\% N''$ in the equation above, V'' may be calculated provided V is known, and the amount of oxygen contained in the lungs at the start and at the end of any interval subsequently considered. This has been done for intervals of 5 minutes each throughout a diving period lasting for 105 min using an average value for $\% O_2$, $\% CO_2$ and $\% N$ in the lung air obtained by utilizing all of the analytical data from the experiments involving such determinations. The results are shown in Fig. 12 where also all the analytical oxygen values are plotted in. The curve showing the average oxygen consumption per minute for every interval of 5 min, has been calculated for an animal with an initial lung volume of 500 ml and at a temperature in

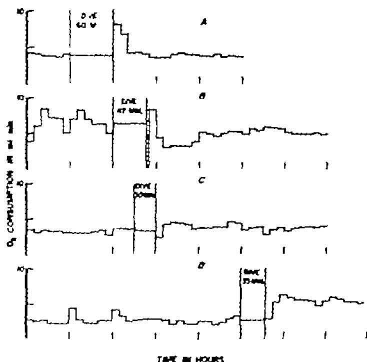


Fig 13 The pre- and post-div consumption of O of the alligator

the range of 21—25 C. It appears that the consumption of oxygen from the lung air would be 0.2—0 4 ml/min during the last 75 min of the dive, i e only 3—6 of the pre-diving oxygen consumption.

Metabolic studies by indirect calorimetry

Pre- and post-dive consumption of oxygen was studied in order to obtain some nformation on the over-all metabolic rate during diving A sample o four experiments Fig 13 A, B C and D) has been chosen to represent these experiments. The oxygen consumption is given for intervals of 10 min each. The br k n lin drawn through the period of submersion in eacl case is the mean oxygen consumption in the pre-diving period for that particular experiment In som of the experiments it was not possible to obtain a very constant rate of oxygen con mptic n n the pre-diving period. Such an experiment is shown in Fi 13 B This wa sometimes due to activity on the part of the animal, especiall I n the experiments were carried out at temperatures above 25 C In m st ses, however a period of constant oxygen consumption could be recorded I we postponed the dive for several hours.

Upon emersion the llig t r sometimes refused to breath for several min, i e., be kept on di ng after he had access to air An example is given in

Fig 13 B In most of the experiments the excess intake of oxygen after a dive was less than would have been expected if the aerobic and anaerobic processes during diving together maintained the metabolic rate of the pre-diving period (Fig 13 A, B and C) It happened, however that the post-dive consumption of oxygen was high, and that t stayed high for hours after the dive was completed.

Discussion

In the present study t was found that the oxygen depots of the alligator were almost exhausted when the animal had endured experimental diving for about two hours at temperatures around 25 C.

Hence, the cardinal question to be answered in order to explain why the diving animals are able to remain submerged for such a long time, is concerned with the oxygen budget of these animals, since an adequate supply of this gas is indispensible for survival. It seems appropriate therefore to begin the discussion with an evaluation of the oxygen depots of the alligator

It is generally assumed that the three main stores of oxygen in a diving animal are the lung air th blood and the muscles. The muscles of the alligator are very pale. This characteristic suggests that they d not contain an appreciable amount of myoglobin, and t is therefore believed that this tissue does not contribute much to th total amount of oxygen stored in the alligator body The quantity of oxygen present in the lung air and in the blood of the animals can be fairly well estimated from the data reported in the previous section. For th present discussion a specimen weighing about 3 kg will be considered.

A easonable figure for the lung volume of such an animal would be in the range of 250—300 ml. The extent to which the alligator inflates its lungs during inspiration may however vary widely from one respiratory cycle to the next. In order not to under-estimate this oxygen store of the alligator the larger value will be used here although the animals frequently did reduce this oxygen depot themselves by exhaling a part of their diving air under water

Immediately after an inspiration the alveolar air may contain roughly 17 Vol % of oxygen. Basing this estimate on the optimal condition that submersion took place just after an inspiration, and that no air was exhaled during the dive, the lung air would constitute a depot of 51 ml of oxygen.

The blood volume of an animal this size would probably be around 150 ml (T ble I) and one may assume that the oxygen capacity of the blood would be close to 10 Vol % (Tabl II)

I case all of this blood was arterialized to full saturation, it would contain 15 ml of oxygen. However in any vertebrate the greater part of the blood is at any one time in the venous state, and this situation is especially exaggerated in the diving animals. Besides, Figs. 6, 7 and 8 reveal that even in th resting period, the arterial blood is not saturated to more than 60—80 %

of its full capacity. This high degree of unsaturation of the arterial blood may well be due to the fact that the reptilian lung is greatly inferior to the mammalian with respect to facilitation of gas exchange between the alveolar air and the blood. Large areas of the surface of the reptilian lung lack a respiratory epithelium, and are poorly supplied with blood (Wolf 1933). A fair estimate of the amount of oxygen stored in the blood may be obtained if one assumes that 25 % of the blood is arterialized to contain 7 Vol % of oxygen, and that the remaining 75 % is venous with an average oxygen content of 4.5 Vol %. According to these considerations the oxygen depot of the blood of the alligator discussed amounts to about 8 ml, and the total quantity of oxygen available upon submersion can therefore hardly have been much more than 60 ml.

A reasonable figure for the resting oxygen consumption in this animal under the conditions specified would be 4 ml/min. The oxygen stores of this alligator would therefore be completely depleted after about 15 min of quiet diving, provided that the aerobic metabolism remained unchanged at the prediving rate. However, in several experiments, the alligators recovered from dives lasting for 60 to 120 min. Hence, there is an enormous discrepancy between the expected diving time, and the diving performance actually exhibited. This outstanding ability to endure prolonged diving in spite of the insufficiency of the oxygen stores calls for profound metabolic adjustments.

Several authors (Dill and Edwards 1931, Elsner 1960) have suggested or implied that the natural divers are able to remain under water for such long periods of time due to an anaerobic shift of the metabolism, thus, making up for the oxygen deficiency by a large increase in the anaerobic processes. However, even if the anaerobic metabolism should constitute the largest source of energy during protracted diving, a certain minimal supply of oxygen will nevertheless be essential in this period. The oxygen stores will have to last until emergence is allowed in order for the animal to survive, for, regardless of any metabolic change, it is clearly impossible to maintain tissues which are susceptible to irreversible damage from oxygen deficiency, such as the heart and especially the brain, in the complete absence of this gas. Therefore, the ability of the alligator and any other of the natural divers to perform prolonged diving and survive must be explained, not simply by a metabolic change towards anaerobiosis, but in terms of mechanisms which operate to make the stores of oxygen last throughout the period of asphyxiation. The development of these mechanisms to a high degree of perfection is probably the characteristic which distinguish the natural divers among the animals, and enable them to endure long-time submersions so successfully. The modifications in physiological function described in the previous section are believed to reflect the operation of the oxygen-saving mechanisms, and in the following, these various adjustments and their significance for the diving animal will be considered.

The physiological adjustment to diving most frequently described is the development of a conspicuous bradycardia. This phenomenon is of universal occurrence among the diving animals.

The diving bradycardia is also the most prominent change in the electrocardiogram of the diving alligator as displayed by the ECG-tracings shown in Fig 2. After 14 min of diving the time elapsing from one point in the cardiac cycle to the corresponding point in the next was 6.62 sec as compared to 1.30 sec in the pre-diving period. Eighty-seven per cent of this bradycardia was due to a prolongation of the T—Q segment from 0.34 sec to 4 98 sec. As a dive continues the heart rate may drop to 2—3 beats per minute (Fig 3 A) During this extreme bradycardia the heart spends a progressively increasing part of each cardiac cycle in diastole, *i. e.* it is essentially the T—Q segment of the ECG which becomes further prolonged in the long dives.

It is well known that the heart rate slows down in response to stimulation of the vagal nerves because of a modification in the pacemaker rate. As early as 1899 RICHET found that atropinized or vagotomized ducks show no diving bradycardia. The latter phenomenon was confirmed by LOMBROSO (1913) In the one experiment in which the right vagus was cut in an alligator a diving bradycardia did not develop Provided the right vagus was mainly distributed to the sino-auricular junction in this individual as is commonly the case in the mammalian heart, this chance finding tends to support the view that vagal influence also in the alligator is responsible for the slow down of the heart rate during diving

The length of ventricular systole, as approximated by the Q—T interval, increased 51 % from 0.98 to 1.50 sec during the same time. Because of the rate-dependency of the Q—T interval (BAZETT 1920) the possibility existed that the protraction of this interval resulted from bradycardia only However the corresponding Q—T = QT/RR interval was found to be more than 25 % increased over the pre-diving value. This means that the slowing of th ventricular systole is due to other factors as well. Because no direct experimental evidence is available, t is at the present time impossible to decid which other conditions contribute to this slowing of the ventricular systole but t may be a reasonable assumption to ttribute it to changes in the nutritional state of the heart, a progressively increasing anoxemia or to the acidosis incurred during diving

The T wave also exhibited large modifications during a dive. The amplitude is generally increased during the main part of th submersion, and the wave may occasionally appear diphasic or completely inverted. The T-wave is the most labile part of an ECG and it is known to be influenced by a wide variety of stimuli including postural changes and changes in the metabolic conditions.

In the literature bearing on the physiology of diving, the bradycardia is generally recognized to be one of the main adjustments to prolonged sub-

mersions, and it is frequently regarded to be of great importance for a successful endurance of such conditions. LOMBROSO (1913) to the contrary reported that vagotomized ducks were still able to dive well. Unfortunately his paper does not give sufficient data on the diving times endured by the animals to form an opinion of the validity of his conclusion. However the significance of the low heart rate for the ability to perform prolonged diving does not seem quite clear. Obviously the oxygen consumption of the heart itself will decrease when the number of contractions per minute becomes reduced with some 90—95 %. This must be assumed to be a significant way of saving oxygen during diving because the heart, as will be discussed below may be the only muscle which receives oxygen while the animal is submerged. Furthermore, it can be seen from Figs. 10 and 12 that the consumption of oxygen from the lung air is much higher in the initial period of the dive before a low heart rate is established, than it is during the remaining period of the submersion. IRVING et al. (1941 b) found in the seal that the earlier the bradycardia develops, the longer time will the animal be able to extend its sources of oxygen. These findings, however do not necessarily imply that the decrease in the oxygen consumption is simply related to the rapidity with which the bradycardia develops. There are, as will be shown, other circulatory modifications associated with diving which are probably far more important than the bradycardia in terms of oxygen saving mechanisms. Knowledge of the proper stimulus required to induce the diving bradycardia would very likely contribute much towards an understanding of the significance of this modification of physiological function.

Considering the data on the alligator it appears from Fig. 3 A that the bradycardia shows a gradual development. This was characteristic of all the involuntary submersions from which recordings were made. Very frequently an initial tachycardia was observed in these dives. The delay in the onset of the bradycardia points towards asphyxiation as the stimulus for the decreased heart rate. On the other hand in most of the free dives, the bradycardia appeared much more rapidly (Fig. 3 B) quite often a low heart rate was established before the asphyxiation could be of significance. This is indicated by the ECG-recordings obtained in the resting state, for during regular breathing the alligator frequently held his breath for intermittent periods of 30 to 90 sec, and during this time no change in the ECG was noticed. In the seal, the bradycardia follows immediately after submersion (SCHOLANDER 1940). These observations may indicate that the bradycardia perhaps is brought about by reflex action in response to the submersion, possibly by a general stimulation of the vagal center.

There remains, however the possibility that the bradycardia is not at all a primary adjustment to diving but that it merely reflects and compensates for some other circulatory change which may be the principal circulatory modification during diving. This alternative explanation of the cause of the

bradycardia will be taken up again during the discussion of the circulatory changes.

In order to serve a physiological purpose, the object of the various adjustments exhibited during prolonged diving must, as has already been emphasized, be to extend the period of time which the limited stores of oxygen would normally last, so that the animal does not perish. A functionally excellent solution of this problem would be to shut off the blood supply to some organs and tissues which are not critically dependent upon a continuous, ample supply of oxygen. This would not only diminish the total consumption of oxygen per unit time markedly but it would leave the oxygen for the more sensitive parts of the organism. There is strong evidence that such a mechanism does operate in the diving animals.

In Figs. 7 and 8, the concentrations of oxygen and lactic acid in the arterial blood have been illustrated in two dives of comparable length. The dive illustrated in Fig 7 was quietly endured, whereas in the one shown in Fig 8, the animal struggled vigorously several times throughout the diving period. As a result of this difference in diving behaviour the lactic acid concentration of the blood rose to 150 mg % vs. 450 mg % respectively in the post-diving period. During the dive, however only a comparatively small increase in the concentration of blood lactate was observed. In both dives the concentration of oxygen in the arterial blood diminished from 6—7 Vol % at the beginning of the dive to approximately 2—3 Vol % just prior to emersion. The loss of oxygen was rapid in the initial part of the dives, and it parallels a similar depletion of oxygen in the lung air (Figs. 10 and 12) However although the one dive (Fig 8) was so uneasily endured, as judged by the struggling of the animal, the rate with which the oxygen was lost from the blood was no greater than in the quiet one. At the end of the dive the animal which showed the high degree of activity during diving displayed a larger concentration of oxygen in the arterial blood than the other These two findings show that there is little, if any exchange of oxygen and metabolites between the muscles and the circulating blood during diving *i. e.* the muscular tissue is not perfused. The constriction of small arteries in the thigh was frequently observed during diving in the experiments in which the femoral artery was exposed. These findings fit well with the observation of SCHOLANDER *et al.* (1942) that the myoglobin of seal muscles is completely reduced at an early stage of a dive when the arterial blood *is still half saturated. Considering that the myoglobin* has a greater affinity for oxygen than hemoglobin has, the only reasonable conclusion to be drawn from these findings is again that the muscles were not perfused while the animal was submerged When one appreciates the large mass of muscular tissue and its high rate of oxygen consumption when active, the shut off of the muscular circulation must be recognized to constitute a most beneficial regulation for the purpose of sparing oxygen so that a submersion may be safely extended. Moreover it is very probable that the

vasoconstriction involves other circulatory beds than the muscular one, for IRVING *et al* (1942) reported that the diameter of the smaller arteries and veins of the mesentery gradually diminished during diving until they became quite bloodless in appearance, and the color of the gut itself showed cyanosis of this organ shortly after submergence. Such a widespread vasoconstriction implies a general increase in peripheral resistance. This is suggested by the increasingly gentle slope of the descending limb of the arterial blood pressure curves in Fig 4 which relates to the diastolic phase of the cardiac cycle, because this observation indicates a retarded rate of emptying of the reservoir of the large arteries. A similar picture could, of course, have been obtained if the stroke volume increased much in order to keep the cardiac output constant. This is less likely however since it calls for a dilatation of the heart up to 15 times.

As a result of the suggested increase in the peripheral resistance, one would expect a marked rise in the arterial blood pressure as soon as the vessels of the various vascular beds involved start contricting That this actually happened is clearly demonstrated in Fig 5 The systolic, diastolic and pulse pressures were consistently observed to increase 60—70 % above resting level. This makes it possible that the bradycardia is reflexly initiated in response to the circulatory adjustments leading to this elevation in the arterial blood pressure. If this is so one is left with the puzzling fact that in spite of the advancing fall in arterial blood pressure throughout the remaining part of the dive, the bradycardia persists or is even developed further (Fig 3 A) There is one plausible explanation for this phenomenon, namely that the bradycardia initially established either by the submersion itself or in response to the elevated pressures, is maintained, and in some cases further developed by an increasing degree of asphyxiation but the complexity of the situation is such that at the present time a final answer to the cause and maintenance of the diving bradycardia cannot be provided

The shut off of the perfusion to the muscular tissues persists evidently throughout the dive, as is apparent from the lactic acid picture of the arterial blood. This calls for an explanation because it is well known that accumulation of lactic acid or carbon dioxide, decrease in the oxygen tension or a fall in pH tend to relax the arterioles. It is necessary to postulate then, that the effect of these local vasodilators is overcome by some other factor presumably of nervous or endocrine character How this is brought about is not known.

Besides reserving the oxygen stores for the brain and the heart, another principle required for the safe extension of under water exposure would be the ability to utilize the oxygen stores to a very high degree and still maintain a state of "useful consciousness" Useful consciousness has been defined to mean that state in which the individual remains attentive and is able to perform useful or purposeful acts (HALL 1949) In the crocodilians it seems likely from their method of catching prey that this feature must be of direct

value, for these animals are known to grasp their victim, pull him under water to drown him, and then again surface in order to feed.

Whereas man usually loses consciousness if the partial pressure of oxygen in the inspired air falls below 60 mm Hg, which corresponds to roughly 8 Vol % at sea level, the alligator stays alert and will immediately surface when this is made possible, after a dive like the one in Fig 10, where the lungs contained only 1 Vol % of O_2, i. e. the partial pressure was only 7 mm Hg STPD At first thought one would expect to see this high degree of oxygen utilization reflected in a corresponding increase in the CO_2 concentration of the lung air and in corresponding changes in the content of oxygen and carbon dioxide in the arterial blood. The arterial O_2 tension fell in accordance with the diminishing content of oxygen in the lung air as seen by comparing Fig 10 with Figs. 6, 7 and 8. However the rise in the amount of CO_2 in the lung air is always low during diving relative to the drop of the oxygen content, and the *RQ* for the lung air is for the main part of the dive less than 0.5 which is shown in Fig 11 where the corresponding concentrations of oxygen and carbon dioxide have been plotted against each other This situation is still further exaggerated in the blood (Fig 6) where it seems like the concentration of CO_2 does not at all increase after the initial rise during the first 20 minutes of the dive. Here it ought to be mentioned that whereas the analyzer used for the gas analyses easily permits detection of variations of $\pm$ 0.02 % the instrument used for the blood carbon dioxide is limited in accuracy to $\pm$ 1 %. Anyhow the rather slow increase in the carbon dioxide content of the arterial blood and the alveolar air should not surprise us considering that the CO_2 can be stored and buffered in the living organism. In the first place, the diving alligator has only a small amount of stored oxygen from which carbon dioxide can be produced, and a large fraction of the CO_2 formed is probably buffered in the tissues.

The hypothesis that a significant fraction of the carbon dioxide formed is buffered in the tissues during submersions has been experimentally verified in a qualitative way in an earlier study of the diving duck (ANDERSEN 1959 b) and there is no obvious reason to doubt the applicability of the observations discussed above to the alligator

In accordance with the lactic acid picture, the pH of the arterial blood showed its largest drop in the recovery period (Fig 9) There is a concomitant decrease in the blood content of CO_2 to less than 50 % of the normal value which may be seen from Fig 6. This is probably due to an expulsion of carbon dioxide from the blood by the lactic acid.

The physiological significance of the functional adjustments discussed in the previous sections is to delay the exhaustion of the limited oxygen stores of the diver It remains to be evaluated how these adjustments influence the rate of energy metabolism during submergence. The patterns of the aerobic metabolism during diving are revealed by the oxygen consumption from the lung air In Fig 12, the average oxygen consumption in ml/min from this store

is shown for successive period of 5 min each throughout a dive lasting for 105 min. During the last 65 min of this dive, the average consumption of oxygen from this store was 0.3 ml/min, or only about 5 % of the corresponding figure for the pre-diving period at a temperature of 25 C. If the anaerobic processes are going to make up for the deficit they will have to work at an extremely high rate. *The total metabolic rate during diving is most easily* revealed by the study of the oxygen debt incurred by comparing the pre and post-dive oxygen consumptions. A sample of four of these experiments has been reported in Fig 13 A, B C and D In most of the experiments, the post-dive *excess intake of oxygen did not cover the oxygen debt during diving. The one* extreme is pictured in Fig. 13 C where there is only a very slight excess intake of oxygen. On the other hand, it occasionally happened that the O_2 consumption after emersion was enormous (Fig 13 D) In such cases however it seemed like the O_2 consumption was started at an entirely new level, and that it was maintained continually at this high rate for several hours. In the experiments in which the excess uptake of oxygen after the dive did not cover the anticipated oxygen debt, the results inescapably imply a very marked decrease in the total rate of energy metabolism during diving. This finding is in perfect agreement with the observations on the homoiothermic, diving animals studied (SCHOLANDER 1940 ANDERSEN 1959 a)

The work reported in this paper was carried out in the laboratory of Dr P F SCHOLANDER at Scripps Institution of Oceanography University of California, La Jolla, California. I am very deeply indebted to Professor SCHOLANDER for his enthusiastic and critical supervision of my work. At the University of Pennsylvania, Philadelphia, I have benefited greatly from many discussions of the problem with Drs H. T HAMMEL and W. S. YAMAMOTO.

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From The Zoophysiological Institute, University of Lund, Sweden

Ribonucleic Acid and Acid-Soluble Nucleotides of the Early Chick Blastoderm

By

HADAR EMANUELSSON

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Abstract

EMANUELSSON, H. *Ribonucleic acid and acid-soluble nucleotides of the early chick blastoderm.* Acta physiol. scand. 1961 *53* 46—57. — Previous analyses of the nucleic acid content of the embryo area in early chick blastoderms have been extended to comprise also the area opaca. The RNA-variations in the latter are reminiscent of those in the embryo area, but from the 10-hour-stage on their RNA/DNA-ratios are characteristically different. The RNA of the early blastoderm has been analyzed for its nucleotide-composition and also limited analysis of the acid-soluble nucleotides has been undertaken. The last-mentioned fraction is especially distinguished by a high proportion of uracil-nucleotides. To the account is added an analysis of the content of nucleic acids and acid-soluble nucleotides in the unincubated hen egg.

In a previous investigation (EMANUELSSON 1958) the author has shown that during the early development of the chick embryo there occur considerable variations in the concentration of ribonucleic acid and nucleotides in the central part of the blastoderm, the embryo area. These striking fluctuations will naturally give rise to the question of whether similar conditions also prevail in the peripheral part of the blastoderm, i. e. the area opaca. Furthermore, one may ask whether the recorded variations are purely quantitative the radical morphologic changes in the embryo during the investigated period of development suggest that qualitative changes may be involved too. Information bearing just upon these problems is given in the present paper together with

details about the nucleotide content of the early blastoderm. The account is supplemented with data about the occurrence of nucleic acids and nucleotides in the extra-embryonal parts of the hen's egg, the egg-white and the yolk.

Material and Methods

The experimental material was newly laid hen's eggs (White Leghorn, pure breed). Incubation of the eggs was carried out in a thermostatically controlled incubator at 37.5 °C.

When otherwise not stated the data for egg-white and yolk refer to conditions in the unincubated egg. Analyses on the white yolk were made on material collected from eggs incubated for 24 hours in which the white yolk is more easily separated from the blastoderm than in the unincubated egg. Thus the white yolk, referred to in the present paper is more exactly that fraction of the white yolk which is contained in the "nucleus of Pander".

Isolation of the ribonucleic acid (RNA) in the area opaca, egg-white and yolk was carried out as earlier described (Emanuelsson 1958) by extraction with hot 10 per cent NaCl; after hydrolysis in N NaOH the absorption of the RNA-nucleotides was measured in a spectrophotometer at 260 mμ and the values were corrected for the higher extinction shown by the hydrolyzed material as compared with the unhydrolyzed RNA. When analyzing egg-white and yolk it proved necessary first to purify the RNA-hydrolysate by the norite method (see below) before the readings were made.

Determination of the desoxyribonucleic acid (DNA) of the area opaca was also made as earlier described, i.e., after extraction with 10 per cent NaCl and removal of RNA by hydrolysis in N KOH the absorption of alcohol-precipitated DNA was determined spectrophotometrically.

Estimation of the DNA in egg-white and yolk was made according to the Schmidt-Thannhauser method using the diphenylamine test, as quoted by Dische (1955). Owing to inevitable interference from other components in the trichloroacetic acid extract with the test, the latter was supplemented with chromatographic analyses of the nucleic acid derivatives in the extract.

Acid-soluble nucleotides were extracted by homogenization of the tissue material together with the same volume 10 per cent ice-cold trichloroacetic acid. After repeated extraction with 5 per cent trichloroacetic acid the combined extracts as well as the precipitate intended for RNA-analysis were shaken 6 times with ether to remove the trichloroacetic acid. However, before this treatment extracts from yolk and egg-white had first to be filtered through Hyflo Supercel. The ether was removed by aeration, whereupon the nucleotides were purified by adsorption on norite at pH1. After washing the norite with distilled water the nucleotides were released by shaking the norite 5 separate times with a solution of 50 per cent ethanol containing 3 ml conc. ammonia per 100 ml. The method gives a satisfactory yield, although it is known that of the nucleotides DPN and TPN are not released quantitatively.

The purified nucleotides were usually directly hydrolyzed in N HCl and the chromatographed in isopropanol-concentrated HCl-water (97:25:28). The method makes it possible to separate the material into groups of nucleotides with different mother substances (bases) and permits estimation of the total amount of nucleotide-material belonging to each group.

When chromatographing the purified extract of acid-soluble nucleotides troublesome interference with the cytosine-derivatives was experienced from unidentified substances, ninhydrinpositive and with absorption-maximum around 275 mμ. The values, recorded for the cytosine-nucleotides must therefore be considered as very approximate.

Owing to the small amounts of nucleotides usually available a more detailed qualitative and quantitative analysis of the members of each group was considered too uncertain and was consequently omitted. In those cases when identification of a separate nucleotide directly was required the latter was isolated and identified by chromatographing the sample two-dimensionally in propanol-concentrated ammonia-water (60:30:10) and saturated ammonium sulphate-isopropanol-water (72:2:19) respectively.

All chromatograms in the present investigation have been of the ascending type and were made on Whatman No. 1 papers. When possible they have always included some sort of test-substance (usually RNA-hydrolysate) which was run parallel with the sample to be analyzed. In the chromatograms detection of the spots was made in UV light (max. emission at 260 mμ). The spots were eluted over night with 0.1 N HCl, the different substances being conclusively identified and estimated by reading the eluate in spectrophotometer.

The relative proportions of the different nucleotide groups from the trichloroacetic acid extract were in fairly good agreement with that found earlier when — as described in an earlier publication — lanthanum acetate was used to precipitate the nucleotides.

The nucleotide analyses on RNA refer to RNA hydrolysed in N KOH at 37° C for 1 hour. The alkaline hydrolysate was neutralized with perchloric acid and after removal of DNA by precipitation with acidified alcohol in final concentration of 70 per cent the sample was partly evaporated in vacuo, hydrolyzed in N HCl and chromatographed in isopropanol-concentrated HCl-water as described above. In most instances it proved necessary first to purify the RNA-nucleotides by norite adsorption (see above) before the hydrolysis in HCl. Detection, identification and estimation of the products of hydrolysis in the chromatograms were performed as described for the acid-soluble nucleotides.

Sugar analysis of the white yolk was made according to the phloroglucinol method of v. EULER and HAHN (1946). The absorption of the resulting colored solution was read in spectrophotometer at 422 mμ together with glucose standards.

Nitrogen analysis of the white yolk was made according to BOISSONNAS and HAERLE... (1953).

The cultivation of embryo explants has been described previously (EMANUELSSON 1958). Here should only be mentioned that the culture medium consists simply of chicken-Ringer to which has been added agar-agar + glucose. After 24 hours incubation on this medium explants show poor growth but excellent differentiation.

Results

RNA of the Area Opaca

Analysis of the whole chick blastoderm reveals that not only the embryo area but also the area opaca around it is subjected to considerable changes in the cellular concentration of RNA.

For the embryo area it was earlier demonstrated that during the first 60 hours of development the RNA/DNA-quotient ranges between 2,5 and 10.

It is now found (after analysis of the area opaca from various developmental stages between 0 and 60 hours of incubation) that also within the area opaca there exists a similar variation, the values ranging between 3 and 10. The low values (3—4) for the latter region are attained at about the 10-hour and the 20-hour-stage respectively. At other stages the values are mostly high (around 8) and it is especially noted that from the 35-hour-stage until the 60-hour stage the RNA/DNA level lies fixed at the value 8. On the two occasions when

Table I. RNA composition of early chick blastoderms

Stage of development	Part of blastoderm	Molar proportions A	G	C	U	G/C	$\frac{G+U}{A+C}$	$\frac{Pu}{Py}$
0 hours	Whole blastoderm.	10.0	12.1	10.8	7.0	1.12	0.92	1.24
18 hours	Embryo area	10.0	14.7	11.0	8.7	1.34	1.11	1.11
18 hours[1]	Embryo area	10.0	10.2	10.6	9.4	0.96	0.94	1.01
	Area opaca	10.0	15.6	15.7	15.6	1.0	1.22	0.82
24 hours	Embryo area	10.0	15.5	14.0	12.0	1.11	1.15	0.98
	Area opaca.	10.0	15.2	13.4	11.6	1.13	1.15	1.01
18+24 hours[2]	Explanted embryo area	10.0	14.5	13.3	11.6	1.09	1.12	0.98
48 hours	Embryo area	10.0	16.5	13.2	9.0	1.25	1.10	1.19
	Area opaca.	10.0	15.8	11.0	8.1	1.44	1.14	1.35
72 hours	Whole blastoderm.	10.0	18.3	12.3	10.5	1.49	1.29	1.26

Abbreviations used: A = adenylic acid, G = guanylic acid, C = cytidylic acid, U = uridylic acid, Pu = purines, Py = pyrimidines.
[1] Cooled for 5 days at + 2° C.
[2] 18-hour-embryos cultivated for 24 hours in vitro.

the quotient drops to its lowest values for the area opaca, marked decreases are also met with in the embryo region. Otherwise it is apparent that during the investigated period of development the two parts of the blastoderm show fundamentally different trends of development as regards the RNA/DNA-quotient: in the embryo region it is gradually decreasing to a low level, in the area opaca it is finally stabilized at a high level. From the 10-hour-stage on this difference is quite obvious.

No attempts have yet been made by the author to estimate the amount of DNA per cell in the area opaca. However as it has been found for the embryo area that the DNA-content during the first 24 hours will reach values twice the amount which is recorded after that period (EMANUELSSON 1961) it is not excluded that at the same stages of development there is a similar variation in DNA-content in the area opaca too. Consequently it cannot be definitely argued that the RNA/DNA-quotient of the area opaca truly reflects the average amount of RNA per cell, but even if it were calculated with the aforesaid DNA-changes in this region too, the RNA variation during the first 30 hours would nevertheless be appreciable.

Besides a normal variation in the RNA/DNA-quotient in the blastoderm cells it is found that for early stages (10-20 hours of development) interruption of incubation when already in progress will soon lead to a decrease of the same quotient both in the embryo region and the area opaca. This condition was observed when investigating the formation of anidian blastoderms, i. e., blastoderms in which the embryo area is more or less degenerated but the margin (area opaca) still continues to develop essentially normally. This phenomenon may be

evoked if during the earliest development the eggs are cooled down and kept at —3° C for some days and then are reincubated at normal temperature (+37,5° C) (GRODZIŃSKI 1934). A less drastic treatment, involving cooling for some days at + 2° C, has been found to cause morphologic disturbances of only a minor part of the treated embryos during the continued incubation. The cytologic disturbances, however, are common in all the embryos, mainly reflected in abnormally extended anaphase chromosomes.

Now biochemical analysis of this embryo material (18 hour blastoderms, cooled for 5 days at + 2° C) shows that RNA/DNA-quotients as compared with the initial conditions before cooling have decreased with 29.5 per cent (embryo area) and 24 per cent (area opaca). Even if about half this decrease must be ascribed to DNA synthesis taking place during cooling of the eggs, the other half undubitably represents a real loss of RNA, which may be estimated to between 5 and 10 per cent of the total RNA-content in each region. In the embryo area a 14 per cent increase of the normal level of free nucleotides was simultaneously registered. Qualitative changes of RNA have also been recorded for the cooled blastoderms (Table I).

Nucleotide Composition of RNA from Early Chick Blastoderms

The analytic data are assembled in Table 1. For each stage they represent average values from 4—6 separate analyses, each comprising material from 50—200 blastoderms. They have all been in close agreement.

In the unincubated blastoderm there are still no differences in RNA-composition between the central embryo area and the peripheral area opaca. It is to be noted, however, that at this stage, when development is at a standstill, even if mitotic activity is not altogether lacking the proportions between the RNA-nucleotides are markedly different from what are found in more advanced stages in active growth.

When incubation has started a slight but scarcely significant difference in RNA-composition between the two actual regions of the blastoderm is indicated after 24 hours. After an additional 24 hours the difference is clearly pronounced, the Pu/Py ratios being markedly dissimilar. A common feature for both regions during the course of development is the gradually increasing proportion of guanylic acid in the RNA.

Closer inspection of the found nucleotide ratios will disclose certain regularities in their variation, which reasonably should have connection with actual conditions of cell-multiplication prevailing in the analyzed regions. In the present case it seems to the author that of the molar relationships included, the G/C-ratio, i. e. the relation between guanylic and cytidylic acid, most strikingly reflects the mitotic activity in the investigated material. Thus high values of this ratio apparently correspond to high mitotic activity, whereas low values denote the reverse condition. The observed connection is clearly indicated

Table II. Acid-soluble nucleotides of early chick blastoderms

Stage of development	Part of blastoderm	Nucleotide composition (as moles per 100 moles nucleotide)			
		A	G	C	U
0 hours	Whole blastoderm.	47.4	8.0	10.4	34.2
24 hours	Embryo area	43.0	8.3	14.7	32.0
	Whole blastoderm.	43.2	8.7	5.7	42.4
48 hours	Whole blastoderm.	63.0	16.9	8.2	11.9
72 hours	Area opaca	52.6	11.8	6.5	29.1
	Whole blastoderm.	56.5	19.5	7.5	16.5

Abbreviations used A = adenine nucleotides, G = guanine nucleotides, C = cytosine nucleotides, U = uracil nucleotides

by the analytic values obtained from ordinary 18-hour-embryos and 18-hour embryos cultivated in vitro on a poor culture medium totally lacking in proteins. The former embryos show a high mitotic index, higher e. g than in the succeeding more advanced 24-hour-embryo whereas in the explants the mitotic activity is low after 24 hours incubation. Nevertheless a differentiation has occurred in them which is nearly equivalent to that in normal embryos of corresponding age.

Except for the 72 hour-blastoderms the investigated material has shown a composition of the RNA which is in fairly good agreement with "Chargaff's rule" i. e., the bases with 6-amino groups and those with 6-keto groups occur in approximatively equal amounts (Elson and Chargaff 1955)

Acid-Soluble Nucleotides of the Early Chick Blastoderm

The author has earlier reported the occurrence of a varying level of free nucleotides in the early chick embryo. In the present investigation some quantitative relationships for these nucleotides are presented, which furnish at least a rough picture of conditions prevailing in the early chick blastoderm. Paper chromatography has in all cases been the method of analysis. It is obvious that ion-exchange chromatography would have been preferable, but owing to the difficulties in collecting sufficient material for that type of analysis it had to be left out of consideration.

Analysis of trichloroacetic acid extracts from early blastoderms reveals the occurrence in them of nucleotides which are derivatives of adenine, guanine, cytosine and uracil. The relative proportions of these are given in Table II Each stage is represented by at least 4 separate determinations, which all have been in good agreement. In fact traces of thymine derivatives were also observed in the extracts from the 24-hour blastoderms.

When reading the table it should be observed that the proportion of cytosine

derivatives stated here is approximative only and presumably too high. Furthermore, it should be stated that it has been impossible to obtain the early blastoderms free from adhering yolk granula. These yolk granula together with already absorbed, intracellular yolk material will obviously constitute a factor of dilution. As will be shown below the yolk granula are characterized by a comparatively high amount of uracil nucleotides, so there is reason to suppose that the high values for the uracil derivates, stated in Table II to a certain extent refer to extra and intra cellular yolk (and presumably also absorbed egg-white) material. If that assumption holds true, the values in Table II suggest higher relative amount of yolk material in the earliest developmental stages, moreover they would indicate more yolk granula in the area opaca than in the embryo area. Both conditions were confirmed on cytologic examination.

Of the remaining groups of nucleotides included in The Table II namely the adenine derivatives and the guanine derivatives, the former are quantitatively dominating. The absolute amounts are of less interest in the present case, bearing in mind the possible variations in the amount of yolk present in early blastoderms, yet it appears that for both the 48-hour and the 72 hour-stage the concentration of adenine nucleotides is comparatively similar corresponding to c. 110 μM adenine/100 g wet weight. For earlier stages the concentration of adenine derivatives is apparently higher.

As for the mutual relation between the two last mentioned groups of nucleotides it is of interest to record that the lowest proportion of guanine derivatives is met with in the 0-hour-blastoderm, the highest in the 48-hour blastoderm. Thus a resemblance to the variations of the G/A-ratio for RNA from the same developmental stages is evident. The analytic data for normal 18-hour embryos and 18-hour embryos cultivated in vitro are partly incomplete, but they have nevertheless shown that also these stages follow the same pattern with respect to the acid-soluble adenine and guanine derivatives.

Nucleic Acid and Acid-Soluble Nucleotides in Egg-White and Yolk

When dealing with problems connected with the synthesis of nucleic acids in the developing chick embryo it should not be forgotten that in the extra-embryonal parts of the hen's egg there is in fact a significant amount of nucleic acids and nucleotides. The role of these in the development of the chick embryo is, however, still poorly understood.

Fraenkel—Conrat et al (1951) were the first to establish definitely the occurrence of DNA-protein in the egg-white of the hen's egg. Later analyses by Solomon (1957) have demonstrated the occurrence of DNA and RNA, both in egg white and yolk. As owing to high protein-contamination in combination with large extract volumes, the previous analyses of the present author failed to prove the definite existence of DNA in the yolk of the hen's egg, a reinvestigation based on other methods of analysis has been made of the nucleic acid content of the egg-white and the yolk. The values given in Table III were arrived at for

Table III Nucleic acid content and acid-soluble nucleotides of the unincubated Hen s egg

Average weights egg-white	33 g		*Acid-soluble nucleotides*				
yolk	17 g			Nucleotide composition (as moles per 100 moles nucleotide)			
Nucleic acid content	DNA	RNA			A	G	U
Egg-white	160 µg	250 µg	Egg-white		8.1	27.8	64.1
Yolk (blastoderm removed)	50 µg	60 µg	Yolk		9.9	24.1	66.0

Absolute amounts of uracil nucleotides egg-white c. 1.5 µM/100 g, yolk c. 0.6 µM/100 g

Abbreviations used see Table II

unincubated hen eggs. The figures are in tolerable agreement with Solomon s results, but they are somewhat lower throughout. Besides an obvious variation in the DNA-content of the yolk (± 20 per cent) of the investigated eggs, it was found that the DNA is not uniformly dispersed but occurs in notably higher concentration (10—15 times higher) in the white yolk, located directly under the developing blastoderm. Other analyses further revealed that calculated per unit of nitrogen the sugar content of this white yolk is appreciably higher than in the yellow yolk (1 07 µg/µg nitrogen against 0,76 µg/µg nitrogen, in both cases calculated as glucose)

Concerning the DNA of the entire egg-white the present author has registered an apparently significant decrease already during th first day of incubation. The decrease which is slightly higher than that recorded during the second day of incubation amounts to 10 per cent of the whole DNA-content.

Investigation of the nucleotide content of the extraembryonal parts of the hen s egg has been made on similar lines as for th blastoderms, i. e., as an analysis of the proportions of the principal types of nucleotides. The values are given in Table III. Cytosine nucleotides are not included in the table as the recorded amounts are quite insignificant.

Separate analysis of the nucleotide content of the white yolk indicates much higher concentration of adenine nucleotides than in the yellow yolk and a dominance of adenine nucleotides over guanine nucleotides which is quite different from the conditions which prevail in the yolk as a whole. The greater part of the adenine nucleotide fraction is composed of ATP

Discussion

Changes of th RNA/DNA-quotient in the area opaca of the early chick blastoderm at about the same time as the occurrence of rather similar changes in the embryo area bear evidence of a fairly similar character of growth in both the regions. From the 10-hour-stage on, however it is clear that the ratio in question

is constantly higher in the area opaca. This condition together with the fact that the periods with decreased RNA/DNA-ratios apparently are not quite synchronous for the two areas will explain the fact that already during the first 30 hours of incubation important ratio-differences may occur at certain stages, e. g., the 18-hour-stage. A more decided deviation is otherwise not evident until the second day of incubation.

For the 18-hour-stage the ratios in fact indicate a RNA concentration in the area opaca about twice as high as that in the rest of the blastoderm. It seems probable that the different sensitivity towards external disturbances which at this stage is displayed by the two regions is connected with these ratio-differences. From the author's experiments it is obvious that factors which affect RNA synthesis of the embryo cells, e. g. extreme temperatures, added steroids etc., have a more pronounced effect upon the embryo area than upon the area opaca. The resulting effect may then be either morphologic disturbances or intracellular changes, visible as chromosome disturbances.

The lack of a functioning vascular system for the rapidly thickening embryonal area will possibly place the nutrition — and with that also the ability to RNA synthesis — in a more exposed position for that region in the more peripheral parts there is still intimate contact with the underlying food reserves. The decrease of the RNA/DNA-ratio in blastoderms in which incubation is interrupted by cooling indicates RNA-deficiency as a plausible cause in the formation of anidian blastoderms. In these the degenerative changes — as mentioned — affect the embryo region but not the area opaca, at least not the outer part thereof. Even if now both the actual regions of the chick blastoderm will show a RNA-decrease during cooling, it seems probable that just the cells of the embryo area with their markedly lower RNA/DNA ratio, will sooner reach a relation between RNA and DNA which is no longer consistent with normal growth and differentiation at the stage in question.

The dominance of the adenine and cytosine components, shown by the RNAs of the embryo area and the area opaca, is apparently a common feature for RNA of animal tissues (MAGASANIK 1955). On the whole the ratios for the components reflect about the same pattern which according to the latter author is found for chicken liver namely A G C U = 10.0 17.1 13.6 10.6. The ratios reported for RNA from 48- and 72 hours-blastoderms are, however markedly different from values obtained for RNA isolated from the area opaca of 40—60-hour blastoderms by Kirby's phenol method (Emanuelsson 1960). The following ratios were then found A G C U = 10.0 22.8 16.3 15.8. Even if the present analyses do not refer to exactly that stage, a comparison may yet be drawn which shows that the last mentioned values indicated a higher G/A-ratio (2.28 against 1.58) and a lower Pu/Py-ratio (1.02 against 1.35) than found here for practically equivalent material.

Now it is a well-known fact that RNA-preparations isolated by different procedures vary greatly in composition (MAGASANIK 1955) and it has just been

shown by YAMANA and SIBATANI (1960) and SIBATANI et al. (1960) that phenol-released RNA does not represent the total RNA-content of a tissue but a major fraction thereof, metabolically little active and with a high guanylic acid content. It is presumably synthesized at about the same rate as DNA, i. e., duplicated only once during the mitotic cycle. Left behind in the phenol phase — and thus actually missed in the isolation procedure — there remains a metabolically active RNA-fraction with low guanylic acid content.

The varying guanylic acid content of the RNA recorded for the blastoderms here in Table I seems quite consistent with the assumption of two metabolically different classes of RNA. The increasing guanylic acid ratio of the more advanced stages indicates a diminished proportion of metabolically active RNA which is just to be expected, bearing in mind the decreasing rate of cell-multiplication and the proceeding cell-differentation.

Also the variations of the G/C-ratio with the actual state of cell-multiplication in the embryo are now more clarified, but it is obvious that a definite evaluation of this ratio cannot be made until complete data about the composition of the two classes of RNA are available. Yet it will seem from the analytic values reported by SIBATANI et al. (1960) that the higher G/C-ratio is shown by the metabolically less active RNA.

For the area opaca an interpretation of the present analytic values in accordance with the suggestions proposed above would suggest higher mitotic activity and higher proportion of metabolically active RNA than in the adhering embryo area. It seems very likely that this is in conformity with the actual conditions.

The absolute amount of acid-soluble nucleotides and the prevailing relations between adenine and guanine nucleotides found in the present investigation correspond fairly well to data arrived at for rapidly growing animal tissues (SAUKKONEN 1956). The high proportion of uracil derivatives is, however, a special characteristic of the early blastoderm and is reasonably associated with the presence of uracil derivatives in the egg-white and the yolk. In 15-day-old chick embryos the proportion of uracil derivatives among the acid-soluble nucleotides is apparently much lower (LU and WINNICK 1955). It is generally acknowledged that the avian liver is rich in uracil compounds. However in the present case it should be especially pointed out that STROMINGER (1954, 1955) has isolated from the hen's oviduct several types of uridine and guanosine nucleotides, representatives of the two groups, which dominate in the egg white and the yolk. In the chromatograms of the oviduct extract the former group was dominating, it amounted to c. 0.5 μM/g tissue.

Our present knowledge of the role of the uridine nucleotides shows that they are of great importance in transformation processes between different sugar compounds and in synthesis of polysaccharides. It seems justifiable to assume a close connection between the high amount of uracil derivatives in early blasto-

derms and the established condition (SPRATT 1951) that carbohydrates is the main source of energy for the chick embryo during the first four days of incubation. In this connection should also be emphasized the peculiarity that the very structure of the yolk — with the white yolk close up to the blastoderm — has obviously secured the embryo a direct supply of energy rich phosphate and carbohydrate for the earliest development.

The intense protein-synthesis in the early blastoderm explains the high amount of cytosine- and guanine-nucleotides, as according to our present knowledge (HOAGLAND 1960) both types play an essential rôle in protein synthesis. The observed similarities in variation between RNA and the nucleotide fraction with respect to the adenine/guanine-ratio permits the assumption of a close connexion between the acid-soluble nucleotides and RNA. That nucleotides may act as precursors in nucleic acid synthesis is actually verified in recent investigations cf. e. g. KHORANA (1960)

At the same time it should be realized that part of the nucleotides in the blastoderm evidently emanate from nucleic acid degradation. Even if decomposition of nucleic acids of the embryo during the isolation procedures is not very likely the very presence of nucleic acids both in egg-whit and yolk and the evident ribonuclease-activity in the blastoderm — indicated by the RNA decrease in the cooled embryos — favors such a view Also the rapid decrease of the DNA of the egg white during the first day of incubation and the observation of traces of thymine-derivatives in the nucleotide fraction of the 1-day-blastoderm give further support for the assumption of degradation of extra-embryonal nucleic acid during the earliest development.

For the adenine nucleotides finally the absence of details about proportions of individual adenine nucleotides, especially ADP and ATP which might have given information about energy conditions in the embryo cells, leaves little more to be stated about them here than a recognition of the fact that they are indispensable in the protein synthesis. The high proportion of ATP in the white yolk reveals a marked dissimilarity in nucleotide content between the two types of yolk, the white yolk being more in accordance with conditions in ordinary animal cells. Here should be mentioned that there exist distinct differences in amino acid content too between the white and the yellow yolk (SCHLENDORFF 1951)

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From the Department of Clinical Physiology and the Department of Surgery I, University of Göteborg, Sweden

Bilirubin, Alkaline Phosphatase and Transaminases in Blood and Lymph during Biliary Obstruction in the Cat

By

Arne Carlsten, Yngve Edlund and Olav Thulesius

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Abstract

Carlsten, A., Y. Edlund and O. Thulesius. *Bilirubin, alkaline phosphatase and transaminases in blood and lymph during biliary obstruction in the cat.* Acta physiol. scand. 1961. 53. 58—67. — The mechanism of hyperbilirubinemia and enzyme disturbances in acute biliary stasis have been studied experimentally in the cat. Obstruction of the common bile duct (I) and retrograde infusion of BSP and bilirubin solution (II) into the common bile duct towards the liver at pressures exceeding the maximal secretory level was performed. From these studies the following conclusions may be drawn. (1) In biliary stasis bile products simultaneously reach the blood through the hepatic vein and the liver lymph. The role of the lymphatic pathways to the accumulation of bile products seems to be minimal. (2) The rise in alkaline phosphatase activity in lymph and blood after biliary stasis is only moderate in the cat. (3) After biliary stasis there is a significant increase in transaminase activity in both blood and lymph.

Already in 1793 Saunders reported the appearance of bile tinged liquid in lymph vessels from the liver. Later studies confirmed these observations and claimed that the main mechanism responsible for the development of obstructive jaundice consisted of a primary regurgitation of bile to the liver lymph vessels from which the bile products reached the blood through the thoracic duct (Mayo and Greene 1929, Shafiroff, Doubilet and Ruggiero 1939,

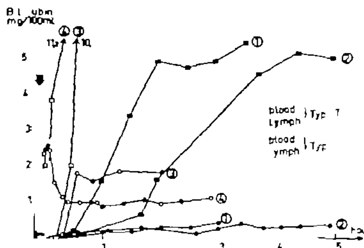


Fig. 1 Bilirubin (total) in peripheral blood and lymph after obstruction of common bile duct (Type I) and infusion of bilirubin solution (Type II) Arrow indicates onset of obstruction or infusion.

SHAFIROFF et al. 1942, 1944 MALLET-GUY et al. 1959) According to others bile products may reach the blood circulation either directly within the liver through radicles of the hepatic vein (HANZON 1952, BRAUER, LEONG and HOLLOWAY 1954) or mainly by the way of lymphatic pathways (BLOOM 1923 GONZALES-ODDONE 1946) These investigators were of the opinion that bilirubin appeared first in the lymph and later in the blood RITCHIE, GRINDLAY and BOLLMAN (1956) studied the mechanism with liver lymph fistulae in trained dogs. They presented evidence showing that "regurgitation of bilirubin into the liver lymph after acute obstruction of the common bile duct was limited to the first 24 hours after onset of obstruction.

The purpose of this study was to evaluate the time relationship of the appearance of bile products in blood and lymph during the initial phase of biliary stasis in the cat. Moreover we have been interested in the pathways of some enzymes related to acute obstruction of the common bile duct.

Material and Methods

Sixteen healthy male and female cats ranging in weight from 2 to 5 kg were used in this study The animals were anaesthetized with ether followed by intravenous chloralose — urethan (50 mg and 100 mg respectively per kg body weight) The thoracic duct was cannulated within the thorax using plastic tube and the lymph collected in heparinized test tubes as described by CARLSTEN (1950) The transthoracic approach required artificial respiration which was maintained by pump-respirator connected to tracheal cannula.

In all experiments blood samples were obtained from the femoral vein and in some series also from the femoral artery and the hepatic vein. The blood samples were taken

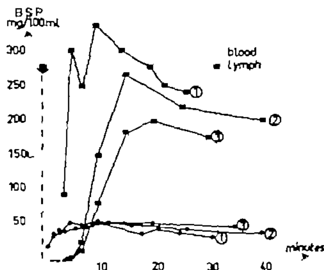


Fig. 2. Sulfobromophtalein (BSP) concentration in peripheral blood and lymph after onset of infusion.

through an indwelling polyethylene catheter. Samples from the hepatic vein were obtained by an ureteral catheter which was inserted from the right external jugular vein. The proper position of the catheter was checked by direct inspection from the abdomen where the coloured catheter easily was visible within a hepatic vein at the liver outflow. Blood and lymph samples were collected simultaneously at different intervals.

In all experiments the cystic duct of the gallbladder was tied before ligation or cannulation of the common bile duct.

Essentially 2 types of experiments were performed.

Type I. Ligation of the distal part of the common bile duct near the duodenal wall.

Type II. Cannulation of the common bile duct (after ligation of the distal part) with a plastic catheter connected to an infusion system which could be adapted to a constant hydrostatic pressure level. By this arrangement bilirubin solution (3 % Merck mad of bile calculi from swine) or sulfobromophtalein (BSP) (5 %) could be instilled at different pressures (300—450 mm H_2O).

Chemical analyses. Direct and indirect reacting bilirubin was estimated by the method of JENDRASSIK and CLEGHORN (1936) and JENDRASSIK and GROF (1938). Alkaline phosphatase activity was determined according to the technique of BUCH and BUCH (1939), glutamic-oxaloacetic acid transaminase (GOT) and glutamic pyruvic transaminase (GTP) according to KARMEN, WROBLEWSKI and LADUE (1955). Sulfobromophtalein (BSP) concentration was determined by the method of SELIGSON, MARINO and DODSON (1957).

Results

In our first experiments blood samples were obtained from both peripheral vessles (femoral artery and vein) as well as from the hepatic vein. In experiments according to type I (ligation of the common bile duct) no significant difference in blood concentration of bilirubin and enzymes could be detected in samples from peripheral or central blood. Only slightly higher concentrations of bilirubin (Fig. 1) and BSP could be observed in samples from the hepatic vein in the infusion experiments within the first period of 30 min (type

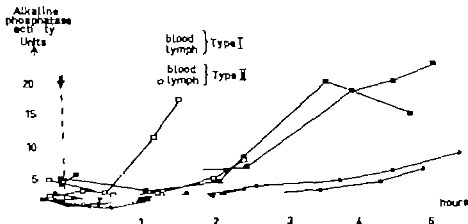


Fig 3. Alkaline phosphatase activity in peripheral blood and lymph after obstruction of common bile duct (Type I) and infusion (Type II) of bilirubin and BSP solutions. Arrow indicates onset of obstruction or infusion.

II) Later the same concentrations were observed in peripheral and central blood.

The lymph-flow per hour from the thoracic duct ranged from 1 4—5 6 ml/kg of body weight (mean 2.9 ml/kg) No increase of the lymph-flow values could be observed in our experiments following biliary obstruction. In some experiments according to type I the maximal biliary secretion pressure was measured This was found to be 280—350 mm H_2O

A. *Studies on Bilirubin Concentration*

Type I Four experiments were performed in this group with occlusion of the common bile duct. Blood from the femoral artery and thoracic duct lymph was analysed with respect to total bilirubin. The results of two typical experiments are shown in Fig 1 The bilirubin values showed a slight increase in blood and lymph during the first hour of biliary stasis in all 4 animals. Thereupon followed a marked increase in the bilirubin concentration of the lymph. In the blood the bilirubin level increased only slowly and reached values out ranging the normal concentration (two tandard deviations above the normal mean value) one hour after the onset of biliary stasis.

Type II In this series 3 experiments were performed with infusion of bilirubin solution into the common bile duct. Two of these are shown in Fig 1 In 2 animals the bilirubin concentration in blood (from femoral artery and hepatic vein) increased markedly already 1 minute after the infusion was started. In the third a significant rise in blood concentration could be shown to appear 35 min after the onset of infusion. The result of this experiment as well as one example with the short ppearance time are plotted in Fig 1 In

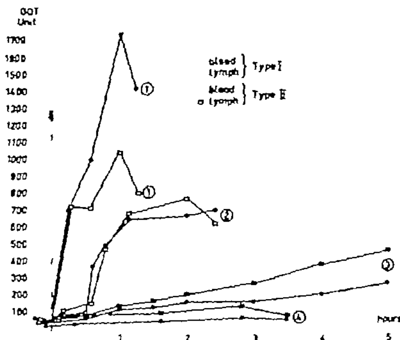


Fig. 4 GOT-activity in peripheral blood and lymph after obstruction of common bile duct (Type I) and infusion (Type II) of bilirubin (2) and BSP-solutions (1). Arrow indicates onset of obstruction or infusion.

only one experiment the lymph flow was sufficient to obtain repeated samples at short intervals. In this instance a sharp rise of the bilirubin concentration in the lymph was observed at 5 minutes after the onset of infusion. Also in these experiments lymph concentrations always exceeded blood values markedly.

B. *Infusion of BSP*

In this group 4 experiments were made with infusion of 5—12 ml of BSP solution at a constant pressure of 450 mm H O (see Fig. 2). We succeded to get repeated lymph samples at short intervals within the first five minutes in two instances. In one experiment only blood from the femoral artery and hepatic vein was collected. Already the first blood samples withdrawn after 1—2 min contained measurable amounts of BSP. In the lymph BSP could be detected after 4—7 min. After the first peak values in blood and lymph the concentration in the subsequent periods declined, the lymph concentration always remaining highest.

C. *Studies on Enzyme Activity*

Alkaline phosphatase. In experiments with simple ligation of the common bile duct (type I) the alkaline phosphatase activity shows an even more delayed increase in blood and lymph compared with the bilirubin values. A significant

Table I Bilirubin, alkaline phosphatase, GOT and GPT in blood and lymph in 10 animals before biliary obstruction (mean values ± standard deviation)

	Bilirubin (mg/100 ml) Total	Direct	Alkaline Phosphatase (units)	GOT (units)	GPT (units)
Blood	0.12 ± 0.04	0.07 ± 0.02	2.8 ± 1.5	65 ± 21	32 ± 15
Lymph	0.12 ± 0.07	0.09 ± 0.07	2.9 ± 1.6	63 ± 26	24 ± 7

rise of phosphatase activity in lymph appearing after 3 1/2 hours with lower values in the blood. In experiments with infusion of bilirubin solution or BSP (type II) the rise of alkaline phosphatase activity appeared somewhat earlier in the lymph in one experiment (see Fig 3)

GOT In experiments performed according to type I a slight rise in GOT activity could be observed in lymph and blood during 5 hours after biliary obstruction. Intrabiliary infusion with BSP or bilirubin was after a short interval followed by a markedly increased GOT activity (within 35 min (Fig 4)) When sufficient amounts of lymph could be obtained also GPT-activity was determined. In these experiments activity curves followed the same pattern as GOT only at slightly lower levels.

Discussion

Bilirubin is transported in the blood to the liver which excretes the pigment in bile. Passage through the liver produces some change in the pigment which is detectable by the direct van den Bergh reaction. The difference between direct and indirect reacting bilirubin is due to the conjugation of lipid-soluble bilirubin (indirect reacting) with glucuronic acid to water-soluble bilirubin-diglucuronide (direct reacting) Both direct and indirect reacting bilirubin were found to be normal constituents of thoracic duct lymph (see Table I) 30 % of which is derived from the liver (Morris 1956, Ritchie Grindlay and Bollman 1959) The water soluble bilirubin-glucuronide probably reaches the lymph in the portal canals by diffusion from bile ducts which, according to Bollman (1951) are richly surrounded by anastomosing networks of lymph vessels, moreover bilirubin-glucuronid and the bilirubin-albumin complex can pass to the lymph via the pericapillary spaces (Fig 5)

Under conditions of biliary obstruction there is a successive rise of biliary pressure which gradually interferes with the passage of bil constituents from blood to the hepatic cell ("retention" of indirect reacting bilirubin) and from the cell to bile canaliculi. In spite of the increased pressure gradient there is still a limited transfer of bilirubin into the hepatic cells and to adjacent bile capillaries and back to the pericapillary space and sinusoids e. g to lymph and blood ("regurgitation" of direct reacting bilirubin) (Brauer et al. 1954)

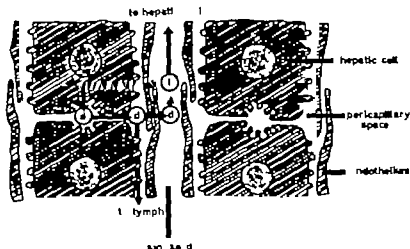


Fig. 5. Schematic drawing of the functional unit of liver lobule. Arrows indicate pathways of bilirubin under normal conditions and during biliary stasis. (i = indirect reacting bilirubin, d = direct reacting bilirubin.) For further details see text.

Our studies on bilirubin concentration in blood and lymph after ligation of the common bile duct (type I) are in accordance with previous investigations (with higher concentrations of bilirubin in lymph). A significant elevation of the bilirubin concentration above normal values seems to be brought about earlier in the lymph. This has led to the concept of the initial transfer of bile constituents to the lymph thus reaching the blood stream through the thoracic duct followed later by direct passage to the blood. These studies did, however not take into consideration the quantitative relationship of the blood and lymph compartments. Assuming that both the thoracic duct lymph and the circulating blood volume received an equal amount of bilirubin at the same time after the onset of biliary obstruction it is obvious that a significant rise in concentration can be traced earlier in the lymph.

From the above mentioned anatomical considerations it is reasonable to assume that bile products regurgitate simultaneously into blood and lymph (via sinusoids of pericapillary space). Our infusion studies present strong evidence in favour of such a mechanism. In fact, infusion of BSP resulted in a shorter appearance time of this test substance in blood from the femoral artery than in thoracic duct lymph. The time necessary for transportation of the indicator from the liver to the thoracic duct can be considered responsible for this short delay (4—5 min). It is therefore reasonable to assume that bile substances reach liver lymph and hepatic vein blood virtually simultaneously. The conclusions may be open to some criticism because they apply only to BSP. Nevertheless the great similarity between BSP and bilirubin has been stressed by many investigators who claim an active transport mechanism connected with a proc-

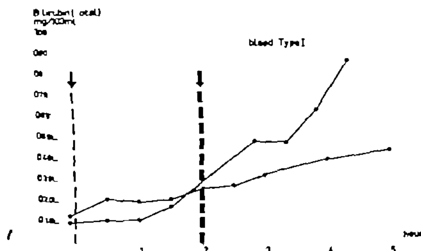


Fig. 6. Bilirubin concentration in peripheral blood (femoral vein) after obstruction of common bile duct (thin arrow). Thick arrow and dotted line (at 2 hours) indicate cannulation of thoracic duct with removal of lymph.

ess of conjugation (WHEELER, MELTZER and BRADLEY 1960) in some way similar to the excretory pattern of bilirubin.

The related experiments do not disclose to what extent the lymphatic system quantitatively contributes to the accumulation of bile products in the blood. We presume, however that these are predominantly discharged from the hepato-biliary system directly into the blood and not through lymphatic pathways. Evidence for this assumption can be found in 2 experiments in which the thoracic duct was cannulated 1 hour after ligation of the common bile duct (Fig 6) Under these conditions the initial rise of bilirubin concentration in blood with intact lymph-flow continued with the same slope after delayed cannulation of the thoracic duct and removal of lymph from the blood circulation.

The mechanism responsible for hyperphosphatasemia in biliary stasis is not fully understood. It is generally suggested that the alkaline phosphatases produced in various tissues are excreted by liver in the bile (FREEMAN and CHEN 1938) The considerations led to the concept of phosphatase "regurgitation" analogous to the mechanism leading to hyperbilirubinemia (FREEMAN, CHEN and IVY 1938, EDLUND 1952) If this was the case then the concentration pattern of phosphatases in blood and lymph in experimental biliary stasis should be similar to that of bilirubin. This is, however not entirely the case in our experiments in which minor differences could be observed with a delayed rise in phosphatase activity compared with bilirubin concentration values. CANTAROW STEWART and McCOOL (1936) and FLOOD GUTMAN and GUTMAN (1937) found only a slight rise in the phosphatase activity after chronic obstruc-

tion of the common bile duct in the cat. They considered this rise to be negligible in comparison with the marked elevations regularly observed following obstruction of the common bile duct in the dog and in man. These authors claimed that this difference was due to excretion of serum phosphatases through the kidney They found that the urine of the cat unlike that of man and the dog, normally contained considerable amounts of alkaline phosphatases. It is somewhat surprising to find such a moderate increase of the phosphatase activity after biliary obstruction and on infusion with BSP or bilirubin solution in our experiments. To some extent this may be explained by renal excretion of phosphatase from the blood. On the other hand not even the lymph values are very impressive and these are not immediately influenced by renal activity The actual experiments are, however limited to observations within 5 hours.

Biliary obstruction could be shown to be followed by steadily increasing GOT activity in blood and lymph. In the infusion experiments an early and even pronounced elevation in GOT activity could be shown to appear in both lymph and blood. CHINSKY SHMAGRANOFF and SHERRY (1957) CHINSKY and SHERRY (1957) and DUNN MARTIAS and REISSMANN (1958) among others have shown that GOT is excreted in the bile in man and in the dog but there is no renal excretion. The very marked rise in GOT activity in the infusion experiments can hardly be explained alone by regurgitation of GOT from the biliary tree. The liver cells with their high content of transaminases must have contributed to this mechanism, the only possible explanation being liver cell damage (BRAUN, PAPP and HORVÁTH 1959) which in our experiments in some way must be related to a toxic effect of the highly concentrated dye (5 %) or bilirubin (3) In 3 control experiments with infusion of saline (0.9 %) and Dextrane (Macrodex) into the common bile duct no sharp rise in transaminase activity could be detected Interference of the high BSP and bilirubin concentrations on the method of GOT determination could be excluded in various controls.

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From the Departments of Physiology and Clinical Biochemistry
Kungl Veterinärhögskolan, Stockholm 51 Sweden

The Effect of Chlorpromazine on the Glucose Metabolism in Different Parts of the Goat Brain

By

S. Larsson

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Abstract

Larsson S *The effect of chlorpromazine on the glucose metabolism in different parts of the goat brain.* Acta physiol. scand. 1961 53 68—74. — Small doses of chlorpromazine were given parenterally to adult goats. Different parts of the hypothalamus and of the cerebral and cerebellar cortici as well as of the posterior and anterior pituitary were then incubated with generally labelled ^{14}C glucose. It was found that the respiratory exchange as well as the ^{14}C lactic acid formation was depressed by the drug in the hypothalamus and in the posterior pituitary The formation of amino acids from exogenous glucose was also inhibited in the same areas after administration of chlorpromazine. The fate of ^{14}C-labelled glucose was essentially unaffected by chlorpromazine in the cerebral and cerebellar cortici.

In series of experiments the fate of ^{14}C-labelled glucose in different parts of the brain and in the pituitary has been studied with the use of a technique for quantitative paper radio-chromatography (Beloff Chain *et al.* 1959, Chain Larsson and Pocchiari 1960 Andersson Larsson and Pocchiari 1961 and Larsson 1961) Thus, it has been found that the formation of amino acids from exogenous ^{14}C glucose is comparatively high in the brain and in the pituitary compared to other tissues. In addition, the different parts of the brain have different metabolic patterns, both quantitatively and qualitatively

As chlorpromazine has been found to accumulate in certain parts of the brain, namley the hypothalamic area (WASE, CHRISTENSEN and POLLEY 1956) the present study was undertaken to see if this substance also caused quantitative or qualitative changes in the metabolic pattern in different parts of the brain.

Material and Methods

Generally labelled ^{14}C glucose was obtained from the Radiochemical Centre, Amersham, England. The radioactive material was diluted to give a specific activity of 20 μC per mg

The composition of the incubation medium was as follows NaCl 0.098 M, KCl 0.027 M, $MgSO_4$ 0.0012 M, KH_2PO_4 0.0004 M, Na_2HPO_4 0.0175 M, and radioactive glucose 0.0056 M—3.6 mC per mM at pH 7.3.

Adult female goats were used for the experiments. The animals were injected with 6.0—7.5 mg Hibernal, LEO (chlorpromazine) intravenously. The dose corresponded roughly to 0.25 mg per kg body weight.

About 15 min after the injection the animals were killed by decapitation. The injected drug caused mild to moderate sedation.

The animals were killed by decapitation after which the brain and the pituitary were dissected out as quickly as possible. The following tissue samples were taken for incubation

1. Periventricular hypothalamic tissue (PV)
2. Ventromedial hypothalamic tissue (VM)
3. Tissue from the cerebral cortex.
4. Tissue from the cerebellar cortex, taken medially along the dorsal curvature.
5. Tissue from the posterior pituitary (PP)
6. Tissue from the anterior pituitary (AP)

The exact localization of the samples, except for no. 4 has been given elsewhere (ANDERSSON *et al.* 1961 LARSSON 1961)

The incubation conditions were the same as previously reported (ANDERSSON *et al.* 1961) Thus, the tissue was incubated for 1 hour at 37° C, the gas phase being pure oxygen. After incubation the medium was immediately transferred to paper for chromatography and the tissue treated as described by CHAIN *et al.* (1960) The solvents for the chromatography were also the same as those used by the previous authors. The chromatograms were scanned quantitatively by transistorized version (LARSSON and STRÖM) of the automatic device described by FRANK *et al.* (1959)

The formation of $^{14}CO_2$ by the tissue samples was determined as described by VILLEE and HASTINGS (1949) The insoluble residue after the extraction of the tissue was not studied.

For the statistical analyses the *t*-test was employed. The following symbols were used
- = differences highly statistically significant
- = differences statistically significant (at the 5 % level or better)
- = differences almost statistically significant (at the 5 % level)

Results

Table I gives the values for oxygen consumption, $^{14}CO_2$- and ^{14}C lactic acid formation in the different parts of the brain and the pituitary with and without previous administration of chlorpromazine. From the table it is

Table I The effect of chlorpromazine on the conversion of ¹⁴-labelled glucose into $^{14}CO_2$ and ¹⁴C lactic acid, and the oxygen consumption in different parts of the brain and the pituitary

Results expressed as μg of glucose converted based on the fraction of total radioactivity of glucose incorporated per 25 mg of tissue (wet wt.) after 1 h incubation at 37°C in O in 0.5 ml of medium. Oxygen consumption expressed in μl per wt. unit. Glucose concentration 0.1 %; total radioactivity 10 μC per vessel. Mean values ± s. e. m.

Chlorprom.		PV	VM	Cort. cerebr	Cort. cerebell.	PP	AP
O	0	38.7 ± 3 1 (14)	34.0 ± 3.3 (10)	36 2 ± 3.7 (6)	33.2 ± 4.3 (3)	30.3 ± 3.2 (10)	26.1 ± 2.6 (9)
	+	14.0 ± 0.6 (5)	14.8 ± 0 6 (5)	30.7 ± 2.7 (5)	28.4 ± 3.2 (3)	15.2 ± 2 0 (5)	18.7 ± 2.3 (4)
¹⁴CO	0[1]	11.9 ± 1.0 (14)	10.8 ± 1.9 (10)	10.5 ± 1 4 (6)	10.0 ± 2.8 (3)	8 7 ± 1.6 (10)	2.3 ± 0.5 (8)
	+	5.0 ± 0.6 (5)	5.0 ± 0.7 (5)	10.6 ± 0 7 (5)	9 7 ± 0.5 (3)	5.3 ± 0 4 (4)	1.3 ± 0.4 (5)
¹⁴C-lact. acid	0	102.3 ± 7.2 (13)	89.8 ± 4.3 (10)	103.3 ± 9.7 (6)	100.1 ± 10 0 (3)	72.3 ± 5 1 (10)	30.3 ± 1.3 (6)
	+	60.3 ± 2.4 (5)	67.2 ± 2.0 (5)	81.5 ± 4.9 (5)	84 4 ± 6.3 (3)	42.8 ± 4 0 (5)	17.9 ± 2.3 (4)

values derived from ANDERSSON et al. (1961) except from cerebrum (LARSSON 1961) and from cerebellum (present study)
Abbreviations of tissue samples — see Materials and Methods
Number f experiments within brackets

evident that the drug administration significantly depressed the oxygen consumption ⁴CO₂, and C lactic acid production in the periventricular and ventromedial hypothalamus as well as in the posterior pituitary In the other parts studied there were essentially no changes in these parameters whether chlorpromazine was administered or not.

Table II shows the formation of ¹⁴C-labelled amino acids in the brain and in the pituitary from the ⁴C glucose in the medium, and how chlorpromazine administration influenced this pattern It was found that the drug decreased the formation of all amino acids, except alanine, from the exogenous glucose in the periventricular tissue In the ventromedial hypothalamic samples the inhibition was less pronounced. In the posterior pituitary glutamic acid formation, in particular was decreased by chlorpromazine This was also true for the samples from the anterior pituitary Further in this part the drug

Table II. The effect of chlorpromazine on the conversion of ^{14}C*-labelled glucose into various amino acids in different parts of the brain and the pituitary*

Results expressed as μg of glucose converted based on the fraction of total radioactivity of glucose incorporated per 25 mg of tissue (wet wt.) after 1 h incubation at 37°C in O_2 in 0.5 ml of medium. Glucose concentration 0.1 %; total radioactivity 10 μC per vessel. Mean values ± s. e. m.

Tissue	Chlor prom.	Glutamic acid	GABA	Alanine	Aspartic acid	Glutamine	Total
PV	*0	6.0 ± 0.7 (14)	3.5 ± 0.3 (14)	1.4 ± 0.1 (14)	0.9 ± 0.1 (14)	1.2 ± 0.3 (14)	13.0 (14)
	+	2.7 ± 0.2 (5)	2.0 ± 0.2 (5)	1.4 ± 0.4 (5)	0.2 ± 0.02 (5)	0.3 ± 0.01 (5)	6.6 (5)
VM	*0	4.4 ± 0.6 (10)	2.6 ± 0.5 (10)	1.3 ± 0.3 (10)	0.5 ± 0.1 (10)	1.1 ± 0.2 (10)	9.9 (10)
	+	2.5 ± 0.2 (5)	1.8 ± 0.3 (5)	1.4 ± 0.4 (5)	0.2 ± 0.1 (5)	0.3 ± 0.01 (5)	6.2 (5)
Cortex cerebr	0	6.4 ± 0.8 (6)	1.3 ± 0.3 (6)	1.4 ± 0.5 (6)	0.8 ± 0.2 (6)	1.5 ± 0.4 (6)	11.4 (6)
	+	5.5 ± 0.4 (5)	1.6 ± 0.2 (5)	1.3 ± 0.05 (5)	0.7 ± 0.02 (5)	0.7 ± 0.02 (5)	9.8 (5)
Cortex cerebell.	0	5.0 ± 0.6 (3)	1.1 ± 0.3 (3)	1.5 ± 0.4 (3)	0.7 ± 0.5 (3)	0.9 ± 0.5 (3)	9.2 (3)
	+	4.6 ± 0.4 (3)	1.4 ± 0.2 (3)	1.7 ± 0.1 (3)	0.8 ± 0.1 (3)	0.2 ± 0.02 (3)	8.7 (3)
PP	*0	4.1 ± 0.3 (10) **	1.0 ± 0.03 (10)	0.7 ± 0.2 (10)	0.8 ± 0.1 (10)	0.7 ± 0.1 (10)	7.3 (10)
	+	1.8 ± 0.3 (3)	1.2 ± 0.1 (5)	0.9 ± 0.2 (5)	0.2 ± 0 (5)	0.8 ± 0.1 (5)	4.9 (5)
AP	*0	0.7 ± 0.1 (8)		0.9 ± 0.1 (8)	0.4 ± 0.05 (8)	0.9 ± 0.2 (8)	2.9 (8)
	+	0.2 ± 0.1 (4)	0.2 ± 0.1 (4)	0.3 ± 0.1 (4)	0.1 ± 0.02 (4)	0.4 ± 0.01 (4)	1.2 (4)

* values derived from [illegible] et al. (1961)
** values derived from Larsson (1961)
GABA = γ-aminobutyric acid
Abbreviations of tissue samples — see Materials and Methods
Number of experiments within brackets

caused the appearance of small amounts of γ-aminobutyric acid. In the samples from the cerebrum and the cerebellum essentially no changes were observed after chlorpromazine injection.

Discussion

When ^{35}S-chlorpromazine was administered parenterally into rats, accumulation of radioactivity in the hypothalamic area of the brain was encountered (WASE *et al.* 1956). In the same study it was also found that the drug caused a marked decrease in the phospholipid turnover of the hypothalamus. In the present study with a different approach, it was found that the administration of chlorpromazine in relatively small, therapeutic doses, caused a decrease in the respiratory exchange of the hypothalamic tissue samples as well as of the posterior pituitary. The cortical samples from the cerebrum and the cerebellum were unaffected by the drug in this respect. In the present experiments the metabolic response of chlorpromazine was studied in 'resting' tissue. However MCILWAIN and GREENGARD (1957) found that chlorpromazine was a potent inhibitor of the metabolic response to electrical pulses in isolated cerebral tissue (cortex) in concentrations of 5 to 10 μM. In the present study on cerebral and cerebellar cortices, even if the oxygen consumption had a tendency to be less after chlorpromazine administration, the differences between normal and injected animals were not significant. EMMELINDO *et al.* (1960) studying certain metabolic trends of psychotropic drugs, found that chlorpromazine depressed oxygen consumption in brain slices. According to their description of the experimental procedures it seemed that the authors did not study the tissue in the di- and mesencephalon. WASE *et al.* (1956) found that the accumulation of ^{35}S from ^{35}S chlorpromazine after repeated injections almost exclusively was encountered in the lipid fraction. This was true for the brain as well as for the plasma and for the liver. Further the most pronounced accumulation of the drug in the brain was in the hypothalamic area as was the decrease in phospholipid turnover due to the drug. As mentioned before the present results indicated the most marked and significant depression of the respiratory exchange as well as of the ^{14}C lactic acid and ^{14}C amino acid formation due to the drug in the hypothalamus and in the posterior pituitary. Accumulating results have shown that when in normal animals different parts of the brain were incubated with ^{14}C glucose or ^{14}C fructose appreciable amounts of ^{14}C-labelled amino acids were found in the tissue extracts (CHAIN *et al.* 1960, ANDERSSON *et al.* 1961 and LARSSON 1961). However when regarding the different parts of the brain they will show amino acid formation pattern which is rather characteristic for the part *per se* both quantitatively and qualitatively (previous references). Even the two parts of the pituitary will differ in this respect (ANDERSSON *et al.* 1961 and LARSSON 1961). Generally one can say that this pattern follows that of phylogenetical origin. However it has also been shown that merely the

state of hunger can cause marked metabolic differences even within areas of the same phylogenetical origin (FORSSBERG and LARSSON 1954 1955) In the present study further it was found that the administration of chlorpromazine in general did not affect the fate of exogenous ^{14}C glucose in the cerebral and cerebellar slices but did so in the periventricular and ventromedial hypothalamic samples as well as in the posterior pituitary and partly also in the anterior pituitary As previously discussed, *in vitro* chlorpromazine caused an inhibition of the oxygen consumption (ERNSTING *et al* 1960) In these experiments, however the substance was present in comparatively high concentrations and incubated with the tissue. According to ERNSTING *et al.* (1960) the action of chlorpromazin should be dual, one mode of action being active on certain phases of the respiratory chain, and one on the membrane permeability According to the authors the latter effect should be the most predominant. In the present experiments t was found that in the periventricular tissue the formation of all amino acids analysed here but alanine was depressed by chlorpromazine. In the ventromedial hypothalamic tissue this effect was less pronounced, but still significant. Partly this was also true for the posterior pituitary Thus, if one forms th ratio between the formation of glutamic acid and of γ-aminobutyric acid in the different parts of the hypothalamus and in the posterior pituitary one will find that the administration of chlorpromazine will make the ratio to approach one, from normally being well above this figure

In this connection it is of interest to note that the posterior pituitary behaved like the hypothalamic samples towards th administration of chlorpromazine. Normally" th posterior pituitary has many similarities with the hypothalamic parts regarding the fate of ^{14}C glucose in the incubation medium (ANDERSSON *et al.* 1961 and LARSSON 1961) Thus, in this respect also, there is a good agreement with what is known about the physiological and histological properties concerning this part in relation to certain areas of the hypothalamus. It was therefore not surprising that chlorpromazine was found to affect the metabolism of the posterior pituitary in a similar way as in the hypothalamus.

Partly in contrary to the findings by ERNSTING *et al* (1960) the present study showed that the rate of conversion of radioactive glucose into various amino cid was depressed due to chlorpromazine in the hypothalamus and in the pituitary However they measured the total amounts of the amino cids while in the present experiments the rate of conversion from exogenous glucose has been measured.

It thus seems that t a higher central level chlorpromazine will nhibit certain aspects of the metabolism of the hypothalamus, the posterior pituitary and partly the anterior pituitary In which latter part γ-aminobutyric acid was found. The present study is taken as a support for the theory that the action of a neuropharmaca will substanciate in biochemical changes specific to the part of the brain where the drug acts.

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From the Institute of Neurophysiology University of Copenhagen, Denmark

The Effect of Deuterium Oxide on the Mechanical Properties of Muscle

By

Ole Svensmark

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Abstract

Svensmark, O. *The effect of deuterium oxide on the mechanical properties of muscle.* Acta physiol. scand. 1961. 53. 75—84. — The effect of deuterium oxide on the isometric contraction of frog muscle was investigated. In 99.8 per cent deuterium oxide the twitch force was reduced to 2—70 per cent of the force in aqueous Ringer, the rate of force development to 12—30 per cent, the tetanic force to 61—96 per cent while the latency of force development was approximately doubled. The total duration of the twitch was unchanged. The duration of the plateau of maximum intensity of active state remained unaltered by deuterium while its decline was enhanced. The conduction time of the action potential was not affected by deuterium but the reduction in frequency at which complete tetanus could be maintained indicated an effect on the excitatory processes. It remained undecided whether the reduction in force is due to an effect of deuterium on the contractile elements or on the excitation-contraction coupling.

It is well known that deuterium inhibits many enzymatic reactions (Katz 1960, Kritchevsky 1960). An enzyme associated with muscular activity, adenosine triphosphatase was inhibited by 50 per cent in 90 per cent deuterium oxide as is seen from the appendix p. 83. It seemed of interest to investigate whether this inhibition is reflected in the mechanical response of skeletal muscle. This report deals with the effect of deuterium oxide on the isometric twitch force, the rate of force development, the latency of force development, the duration of twitch, the tetanic force and the duration of the active state of frog muscle.

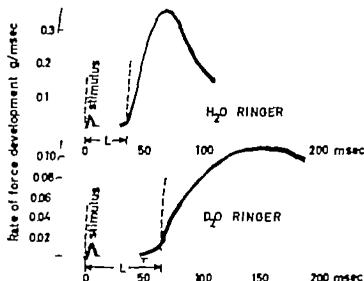


Fig 1 Definition of the latency of maximum acceleration as the time interval L between onset of stimulation and maximum acceleration of the force development. Record of the time course of the rate of force development in isometric twitches at 2° C. Upper curve in H_2O-Ringer Lower curv in 99.8 per cent D_2O-Ringer (M. semitendinosus weight 26 mg length 18 mm stretched 10 per cent above equilibrium length.)

Methods

The experiments were made on the semitendinosus muscle. The frogs (*Rana esculenta*) were curarized (50 μg d-tubocurarine per g frog) and one belly of the muscle (15—40 mg) with maximum diameter of 0.5 to 1 mm was placed in a thermostated chamber t 2° C and bathed Ringer solution with either water or 99.8 per cent deuterium xd as solvent. Th composition of the Ringer was NaCl 115 mM KCl 2.7 mM, Cl_2 1.8 mM, sodium phosphate buffer 2.5 mM pH 7.3 and d-tubocurarine 50 μg per ml.

The muscl was stimulated from end to end or to reduce the conduction time, transversely by multielectrode assembly extending over the entire length. Two assemblies each consisting of fi e platinum lectrodes of alternate polarity were placed on each side at a distance of 4 mm from th axis of the muscle. The *stimuli* were supramaximal rectangular pulses 1.5 msec in duration. Before each experiment the muscle was stimulated with single stimuli t intervals of on minute for a period of one hour only muscles with constant twitch force within this period were used. During the experiments the same rat of stimulation was maintained. In addition, tetanic contractions were evoked t intervals of ten minutes by trains of 15 stimuli per sec and train duration of 1—2 sec. At this low frequency of stimulation the tetanus was almost complete and maximum force was ttained, the change in force per stimulus being less than one per cent. The low frequency was chosen since at higher frequencies the tetanic force decreased in deuterium oxide.

The isometric force was recorded by a Brush ink writer a an electromechanical transducer (RCA 5734 The sensitivity of the recording system allowed a change of 100 mg to be determined. Th response of the recorder to a stepwise increase in force had rise time of 10 msec for full scale deflection, and at the low temperature used

Table I. Rate of deuterium exchange with muscle hydrogen at 0° C. A semitendinosus muscle (19.5 mg) was incubated with 50 per cent D O-Ringer for 1 hour at 20° C and immersed in 2 ml water at 0° C. The deuterium concentration was determined in the outer fluid at various time intervals.

Time of exchange minutes	Concentration of deuterium oxide in the outer fluid (per cent)
0.8	0.122
2.1	0.352
5.2	0.447
10	0.482
20	0.485
40	0.493

(2° C) the time course of force development was recorded without essential distortion. The resting length of the muscle corresponded to load of 2 per cent of the tetanic force. T determine the rate of force development with greater accuracy than obtainable from force recordings, the electronically differentiated signal from the transducer wa recorded simultaneously The open loop gain of the differentiator amplifier was one hundred, and the time constant of the differentiating network was 33 msec. To obtain a well defined determination of the latency of the mechanical response the time interval was measured between onset of stimulation and the time t which the slope of the differentiated record of the force development was maximum (L in Fig 1) This interval corresponds to the latency of maximum acceleration of the change of force and is in the following denoted as 'latency' T obtain sufficient measuring accuracy the amplification was adjusted bout identical maximum deflections before and after exposure to D_2O Application of D_2O resul ed in the same relati increase in latencies of the inflection points, of half peak and peak rate of force development. The latency of maximum acceleration was bout twice the latency estimated from the beginning of force development.

The activ state is defined as the load which the contractile substance can just carry without lengthening (HILL 1949) The duration of the 'plateau of the activ stat and its decline during twitch were determined by the quick release method described by RITCHIE (1954 The release (10 per cent of the muscle length) was obtained by electromagnetic displacement of stiff steel wire to which the muscle was attached. The displacement mechanism was released by the stimulus via a time-delay circuit.

Determination of the exchange of deuterium oxide ith muscle water[1] Semitendinosus muscles of about 20 mg were incubated for one hour w th 50 per cent deuterium oxide Ringer at 20° C. After removal of adhering fluid with filter paper the muscle was immersed in 2 ml water at 0° C. After efficient stirring 15 μl samples were cryosublimated and the deuterium content in the cryosublimat was determined in density gradient tube. (For details of the technique see H IDT *et al.* 1954 KRAGH and LINDERSTRØM-LANG 1953.)

[1] These experiments were carried out in collaboration with the late professor K. LINDERSTRØM-LANG, The Carlsberg Laboratory Copenhagen.

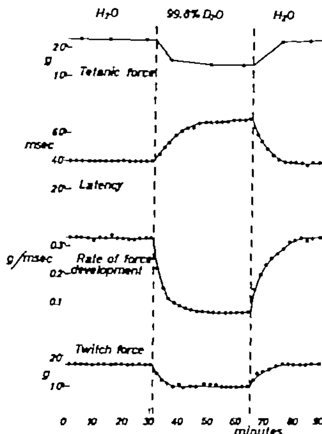


Fig 2. Reversible effect of deuterium oxide on the twitch force, rate of force development, "latency (latency of maximum acceleration) and tetanic force. (M. semitendinosus, weight 28 mg, length 18 mm stretched 10 per cent above equilibrium length.)

Results

1 Rate of deuterium exchange with muscle hydrogen at 0° C An evaluation of the effects of concentrated deuterium on muscle contraction requires that the rate of exchange between deuterium and hydrogen in the muscle is known. To determine this rate the muscle was loaded with deuterium oxide and the rate of back-exchange with water was determined. In the experiment represented in Table I the total amount of deuterium released from the loaded muscle was 15 per cent higher than could be accounted for from a calculation of the water content and the exchangeable protein hydrogen of the muscle. This may possibly be due to deuterium adhering to the surface of the muscle, to a slight swelling or to selective uptake of deuterium at certain sites. Independent of this uncertainty it was, however evident that about 98 per cent of the deuterium appeared during the first 10 min and that the exchange was nearly complete after 40 min. Although the exchange of muscle water and of easily exchangeable hydrogen occurred with half times of less than 2 min, the effects of deuterium on the mechanical response were followed for 1—2 hours to ascertain that also the exchange of more slowly exchangeable hydrogen was completed.

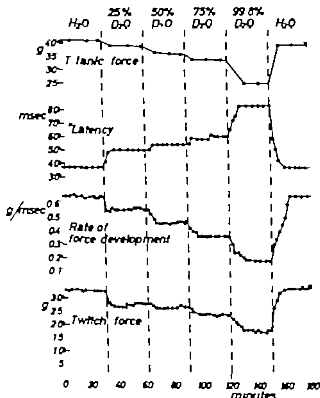


Fig. 3. Effect of different concentrations of deuterium oxide on the twitch force, rate of force development, "latency" (latency of maximum acceleration) and tetanic force. (M. semitendinosus weight 30 mg; length 19 mm; stretched 10 per cent above equilibrium length.)

2. *Mechanical response* In 99.8 per cent D_2O-Ringer the twitch force, the rate of force development and the tetanic force were significantly decreased while the latency increased. These changes took place in the course of the first five to fifteen minutes after replacement of H_2O-Ringer with D_2O-Ringer. The values attained after this initial period remained constant for 3—6 hours as long as D_2O was present. Upon exchange of D_2O-Ringer with H_2O-Ringer the effects of deuterium oxide disappeared within 5—15 min (Fig. 2). The total duration of the twitch was not affected by deuterium.

The changes depended on the concentration of D_2O. The rate of force development decreased nearly proportional to the concentration of D_2O whereas the effect on the twitch force, the 'latency' and the tetanic force was relatively larger at 99.8 per cent than at lower concentrations of D_2O (Fig. 3).

The twitch force averaged 16 ± 1 g (number of experiments 30) in H_2O-Ringer. In 99.8 per cent D_2O-Ringer it decreased to 70 per cent or less in all muscles investigated; in seven muscles the twitch force was less than 15 per cent of the force in H_2O-Ringer. The rate of force development decreased from an average of 0.7 g/msec to 0.03—0.2 g/msec, i.e. by 3 to 8 times. The latency

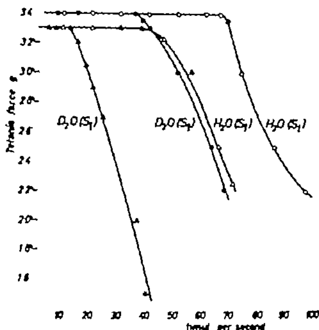


Fig. 4. The decline of tetanic force as function of stimulus frequency in H_2O-Ringer and D_2O-Ringer with end-to-end (S_1) and transverse (S_2) stimulation. (M. semitendinosus; weight 50 mg; length 17 mm; stretched 10 per cent above equilibrium length.)

on the other hand, increased from 30—40 msec in H_2O-Ringer to 60—80 msec in D_2O-Ringer. The tetanic force averaged 24 ± 3 g in H_2O-Ringer (30 exp.) it decreased less than the twitch force (to 61—96 per cent of the value in H_2O-Ringer). Moreover in D_2O Ringer the tetanic force decreased when the stimulation frequency exceeded 12—20 per second whereas it remained independent of frequency up to 40 per second in H_2O-Ringer (fig. 4).

To investigate whether the effect on twitch force, rate of force development and latency were caused by a decreased rate of propagation of the action potential along the muscle fibres the muscle was stimulated alternately by transverse and by end-to-end stimulation. Both in H_2O- and D_2O-Ringer the latency was then reduced to one half with transverse stimulation as compared with end-to-end stimulation but twitch force, rate of force development and tetanic force remained unchanged within the accuracy of recording. In D_2O-Ringer the frequency of stimulation above which the tetanic force declined was 15—40 per second when stimulating through the multielectrode assembly as compared with 12—20 per second with end-to-end stimulation. The corresponding figures for H_2O-Ringer were 70 with the multielectrode and 40 with end-to-end stimulation (Fig. 4).

3. Active state. The active state curve was determined by introducing sudden releases at varying time intervals after the stimulation. In the series of twitches thus obtained the maximum force development was delayed by up to 400 msec and the peaks of these twitches represent points on the active state curve. The

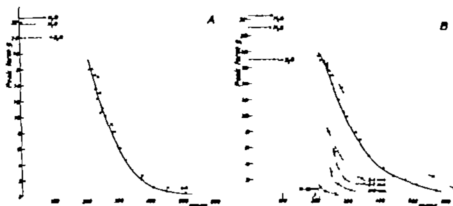


Fig. 5. Plateau and decline of activ state in H_2O-Ringer (●) 99.8 per cent D_2O-Ringer (○) and in H_2O-Ringer after removal of the D_2O-Ringer ( ). The durations of the 'plateau' were determined after more than 30 minutes equilibration with H_2O- or D_2O-Ringer

A An example of the experiments in which the twitch force was reduced by D O to 50—70 per cent of the force in H_2O-Ringer (M. semitendinosus; weight 24 mg; length 17 mm stretched 10 per cent above equilibrium length.)

B An example of the experiments in which the twitch force was reduced by D_2O to 6—20 per cent of the force in H_2O-Ringer The decline of the active state was determined at different times (5—50 minutes) after replacement of H_2O-Ringer with D_2O-Ringer (M. semitendinosus weight 31 mg length 19 mm stretched 10 per cent abov equilibrium length.)

duration of the plateau of maximum intensity of active state was 30—40 msec for a single stimulus and was determined as the time interval from the last stimulus in a tetanus to the time at which the force began to fall. In D O-Ringer the duration of this plateau of maximum intensity of the active state was prolonged by 30—40 msec, possibly due to the increase in the latent period which was of the sam order

As to the decline of the ctive state eleven experiments fell into two groups

A In 5 experiments in which th twitch force decreased in D_2O-Ringer to 70 per cent of the force in H_2O-Ringer the descending phase of the active state curves in D O-Ringer coincided with the lower parts of those in H O-Ringer (Fig 5 A)

B In 6 experiments in which the twitch force was reduced to less than 20 per cent of th force in H O-Ringer (while the tetanic force was reduced only by 15 per cent in average) the decline of the intensity of the active state was progressively enhanced n the course of the first 10—15 min. The time from stimulus to maximum force development remained unchanged (Fig 5 B) The lowered ntensity of the active state persisted as long as D O-Ringer was present and the muscle reversed to normal after a few minutes in H O-Ringer In a number of experiments the decline of active state was determined after the resting force had been either decreased to zero or increased three times. The changes in the course of the decline were small nd could not ccount for the difference bserved between the two groups.

Discussion

The changes in the mechanical properties induced by replacement of water with deuterium oxide may be due to effects on some or all of the elementary processes involved in the contraction of the curarized muscle.

The amplitude of the *action potential* in turtle's muscle is not affected by deuterium (GOODALL 1958) whereas the electroatriogram of frog's heart showed a 10 per cent decrease in amplitude and an increase in latency and in duration of the action potential (KAMINER 1960). In frog's semitendinosus the potential of the resting membrane and the peak of the action potential, as measured with intracellular electrodes, were unaffected by deuterium whereas *the latency and the phase of repolarization increased to about twice their values* in H O-Ringer (BUCHTHAL and ENGBÆK, unpublished). These changes and also the substantial reduction in D O-Ringer of the frequency at which a complete tetanus could be maintained (Fig. 4) suggest that the primary excitatory processes are affected. On the other hand there was no indication that the *conduction time* of the action potential was prolonged by deuterium since the changes in mechanical properties induced by deuterium were not abolished or reduced by using transverse instead of end to-end stimulation.

The changes in active state accomplished by deuterium are probably due to effects on the excitatory processes (or on the excitation-contraction coupling). The decline of the active state was enhanced by deuterium in those muscles in which the twitch force was reduced by more than 50 per cent while it remained unchanged when the twitch force was reduced less. Since the changes were equally reversible after exchange of the D O-Ringer with H O-Ringer the different behavior can hardly be due to injury of some of the muscles. Neither could the difference be due to an incomplete exchange of hydrogen with deuterium as the decline in active state was the same a few minutes after and two to three hours after the application of D O-Ringer. A diminution of the duration of active state by chemical agents has hitherto been described only for monoiodo acetic acid (AUBERT *et al.* 1957). There is no evidence for a true change in duration of maximum intensity of active state by deuterium since the increase observed can be explained in terms of lengthening of the latency period.

The increase in latency of the mechanical response in D O-Ringer is easily explained by the effect of deuterium on the latency of the action potential. The reduction of twitch force and rate of force development by deuterium may be caused *via* changes in the coupling between excitation and contraction and by a direct effect on the contractile substance. GOODALL (1958) concluded from his experiments that the contractile substance was the site of the effect of deuterium since the amplitude of the action potential was not affected. He did not, however, take other changes of the excitatory processes or the excitation-contraction coupling into consideration. KAMINER (1960) suggested that the

excitation-contraction coupling is the link affected by deuterium and not the contractile substance since deuterium does not retard the shortening of the glycerol extracted rabbit's psoas as induced by addition of adenosine triphosphate. The slow diffusion of adenosine triphosphate in the model would, however mask an effect on the rates of the reactions involved and the model experiments, therefore, do not exclude the possibility that the contractile proteins are affected by deuterium. That reaction rates rather than final states are affected is consistent with the present observation of an almost unchanged tetanic force even in experiments where the twitch force and the rate of force development were reduced to one fifth o less by deuterium.

I am greatly indebted to professor F. Buchthal, M. D. and to Mr. P. Rosenfalck, M. Sc. for valuable help and advice. The work was supported by grants from the Danish National Association against Rheumatic Diseases, Copenhagen, and the Muscular Dystrophy Associations of America, Inc., New York City

Appendix

Inhibition of adenosine triphosphatase by deuterium oxide. Rabbit psoas was extracted with 50 per cent glycerol t — 20° C for period of two months. One g of the extracted muscle was homogenized in 5 ml of salt solution (0.16 M KCl and 1 mM $MgCl_2$) One hundred µl of the homogenat were incubated with 0.85 ml 99.8 per cent deuterium oxide containing 0.16 M KCl and 1 mM $MgCl_2$ per liter or with the corresponding aqueous solution t 20° C for 90 min. 50 µl of 50 mM sodium adenosine triphosphate at pH 7.0 were added and the adenosine triphosphatase acti ity determined by electrometric titration t pH 7.0 and 20° C with 0.5 N NaOH. On the averag the adenosine triphosphatase was inhibited by 45 per cent (T ble I)

Table I.

Exp. no	ATP-ase activity (µmoles/min per g muscle) in H_2O	in 85—90 per cent D_2O
1	3.0	1.7
2	2.9	1.8
3	3.0	1.6
4	2.8	1.4

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From the Division of Food Chemistry Royal Institute of Technology and the Departments of Physiology and Clinical Biochemistry Royal Veterinary School, Stockholm

The Loss of Added Lysine and Threonine During the Baking of Wheat Bread

By

L. E. Ericson S. Larsson and G Lid

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Abstract

Ericson, L. E., S. Larsson and G Lid. *The loss of added lysine and threonine during the baking of wheat bread.* Acta physiol. scand. 1961 53. 85—98. — Rat growth experiments and microbiological assay methods were used to estimate the loss of added free L-lysine HCl and DL-threonine during the baking of wheat bread. Introductory rat experiments showed that the optimum concentration for growth of L-lysine HCl added before baking was approximately 0 40—0 45 % of the fresh weight of the flour The optimum concentrations of L-lysine HCl and DL-threonine when added together were approximately 0.55 % for the former amino acid and 0.30 % for the latter The loss of added L-lysine HCl during baking was found to be 10—15 % as estimated by animal experiments and 5—10 % as estimated by microbiological assays. The loss of L-threonine (added as DL-threonine) was significantly greater amounting to approximately 40 as judged by the feeding trials and 20—25 % according to the microbiological estimations.

The amino acid compositions of the flour and the bread baked from the flour were also determined

The addition of lysine to wheat bread results in a considerable increase in the protein value of the bread A further improvement can be obtained by supplementation of the lysine-fortified bread with threonine (Hutchinson, Moran and Pace 1959 Ericson 1960) The addition of other single essential amino acids to wheat bread, either al ne or in combination with lysine and threonine, does not seem to improve its protein value as judged by experiments with rats (Ericson 1960) Similar results ha e been obtained with wheat flour (Sure 1953 1957 Deshpande, Harper and Elvehjem 1957)

In our previous experiments on the amino acid supplementation of bread, the amino acids were added to the bread *after* baking (ERICSON and OVERROTH 1959, ERICSON 1960). Numerous investigators have shown that lysine is easily inactivated in food by reactions with carbohydrates and other compounds. Less is, however, known about the stability of threonine. In the present study an attempt was made to estimate the loss of added lysine and threonine during baking, using both microbiological and rat growth assays.

Experimental

Analytical procedures. For the specific determinations of the lysine and threonine contents of the breads and the diets, microbiological procedures were used. Lysine was determined turbidimetrically with *Leuconostoc mesenteroides* P-60 (ATCC 8042) using Difco's Bacto Lysine Assay Medium. The procedure followed that outlined by STEELE *et al.* (1949) with small modifications. Incubation, for instance, took place at 34° C for 24 h instead of at 37° C for 20 h as suggested by STEELE *et al.* This alteration of the incubation temperature was motivated by the publication of SCHIAFFINO, MCGUIRE and LOY (1958) and was found to give a straighter standard curve and more reproducible results.

Threonine was determined with *Streptococcus faecalis* (ATCC 8043) using Difco's Bacto Threonine Assay Medium — again based on the findings of STEELE *et al.* (1949). The tubes were incubated at 37° C for 24 h.

The turbidity of the assay tubes after incubation was determined at 550 mμ in a Coleman Model 11 Universal spectrophotometer both when *L. mesenteroides* and *S. faecalis* were used.

The inoculum used in the assay of lysine with *L. mesenteroides* was obtained by cultivating the organism overnight at 37° C on Bacto Micro Inoculum Broth fortified as described by BOLDINGH and LARSEN (1961). After centrifuging and washing the cells twice in sterile saline, they were suspended in a quantity of saline giving a Coleman transmittance reading of 50 %. Two ml of this suspension were added to 50 ml saline of which 2 drops were used to inoculate each assay tube.

A similar procedure was used for preparing the inoculum for *S. faecalis*, but the turbidity of the washed cells was in this case adjusted to a Coleman reading of 80. Of this cell suspension, a 2 to 50 dilution was made and 2 drops were used per assay tube.

Various conditions for the liberation of the lysine and threonine were investigated. It was noticed that more reproducible results were obtained when the samples (breads and diets) were extracted with ether petroleum ether before being hydrolysed. Furthermore hydrolysis in 3 N HCl gave a complete hydrolysis and also lighter coloured hydrolysate and a smaller amount of insoluble material ("humin") than hydrolysis in 6 N HCl. For the microbiological determinations of lysine and threonine, the following procedure was therefore followed.

About 10 ml of a mixture of equal parts of ether and petroleum ether (Skellysolve F) was added to a known amount of sample, usually 0.4—0.6 g, in a 13 × 150 mm test tube. The tube was shaken carefully and centrifuged until a clear supernatant fluid was obtained. The solvent was sucked off through a capillary. The procedure was repeated three times. Residual solvent was finally evaporated off by placing the tubes in a stream of warm air.

Six ml of approximately 3 N HCl was added, care being taken to rinse the walls of the test tube free from sample and t let the acid moisten the entire content of the tube. The test tube was then sealed, and placed in sand bath at 110° C. After 24 h, the tube was allowed to cool at + 4 C while still standing in the sand, a procedure which due to distillation results in a rinsing of the upper part of the tube. It was then opened and the content poured into a porcelain cup and evaporated to dryness on a water bath. The dark residue was suspended in a small volume of distilled water filtered and washed. The filtrate was again evaporated to dryness, dissolved in distilled water evaporated again and transferred with distilled water to a glass beaker The pH was adjusted to 6.8 ± 0.2 with 0.5 and 0.01 N NaOH. From each sample treated in this way three different dilutions calculated to fall on the standard curve, were prepared for the microbiological determinations. Each dilution, as well as each standard solution, was run in triplicate in these tests.

The more complete amino acid assays that were made on the flour and the non-supplemented bread were performed according to Moore and Stein (cf. SPACKMAN, STEIN and MOORE 1958) The Beckman/Spinco Model MS Amino Acid Analyzer was employed. These samples were hydrolyzed in sealed evacuated ampoules in 6 N HCl t 110° C for 30—50 h, which was the routine procedure used by the group operating the pparatus. T 0.3 g of each sample 10 ml of the acid was added. N attempt was made to find optimum hydrolysis conditions for the various amino acids. Tryptophan and cystine were not determined.

Nitrogen was determined by the Kjeldahl method as described by PERRET (1955) The ammonia formed on distillation was collected in boric acid and titrated with 0.01 N HCl.

Dry weight determinations were carried out after heating the samples at 104—105° C for 24 h and letting them cool in a desiccator over silica gel.

Type of bread and baking conditions. The bread used in the present investigation was pan baked from dough with the following composition

water	1,000 g
wheat flour[1] (70 % extraction)	1,900 g
lard	20 g
roller-dried skim milk (< 1 % fat)	50 g
sugar	10 g
salt	20 g
malt extract	10 g
yeast	100—200 g

The amount of yeast was adjusted according to the total quantity of dough, more yeast being used when the quantity was small.

Dividing, rounding and moulding were done by hand before the dough pieces were put in the pans. Proofing took place t 45 C for approximately 25—50 min t relative humidity of about 85 %. The baking temperature was 190—220° C and the bread was kept in the oven for bout 20—30 min. Each bread loaf had a weight of approximately 400 g.

Before the bread was used for making the diets, it was cut into slices and dried in air at temperature of 25—27° C for 2 days. It was thereafter ground in an Electrolux Assistent bread mill The dry weight of the ground bread was 90—93 %.

When the amino cids were baked into the bread, they were dissolved in part of the water used for making the dough.

[1] Alexandra, Kvarnfirman AB, Stockholm.

Table I. Dry weights, nitrogen contents and amounts of added L-lysine HCl and DL-threonine for the various groups in Experiments I—III

Exp.	Group	Dry weight Bread %[1]	Nitrogen content Bread %[1]	Nitrogen content Diet %[2]	Added L-lysine HCl %[2]	Added DL-threonine[3] %[2]
I	A	90.9	2.00	1.88	—	—
	B	90.5	2.03	1.86	0.12	—
	C	92.1	2.02	1.90	0.25	—
	D	91.0	2.09	1.95	0.50	—
	E	91.4	2.10	2.00	0.80	—
II	A	93.0	2.18	1.97	0.50	—
	B	92.9	2.19	1.99	0.50	0.30
	C	93.0	2.25	2.01	0.80	0.08
	D	92.6	2.26	2.06	0.80	0.15
	E	92.6	2.26	2.07	0.80	0.30
III	A	92.3	2.19	2.03	[3]0.30	—
	B	92.3	2.19	2.04	0.40	—
	C	92.6	2.24	2.03	0.40	—
	D	91.4	2.27	2.08	0.50	[3]0.15
	E	91.4	2.27	2.12	0.50	[3]0.25
	F	91.5	2.26	2.12	0.50	0.25

[1] Expressed as per cent of the weights of air-dried bread.
[2] Expressed as per cent of th fresh weights of the diets.
[3] These amounts of L-lysine HCl or DL-threonine were added to the diets *after* baking. In all other cases the amino acids were incorporated into the dough.

The diets. All diets contained 91 % of ground bread, 3 % of salt mixture, 5 % soya bean oil, 0.5 cod liver oil and a vitamin mixture. The vitamins were mixed with a small quantity of finely powdered bread before they were added to the general mix.

The salt mixture was identical with that of Hegsted / (1941) [illegible] vitamin mixture had the composition given by Harper *et al.* ([illegible] amounts [illegible] ferrous [illegible] vitamins in mg per 100 g of ration were thiamin [illegible] 1.0, [illegible] *calcium pantothenate* 2.0, *pyridoxine* 0.25, *biotin* [illegible] cyanocobalamin 0.002, inositol 10, menadione 0.5 [illegible] cod liver oil supplied approximately 375 I. U. [illegible] .5 I [illegible] per 100 g of ration. These values are [illegible] (Ericson and Overtoom 1959 [illegible] ration of some of the experiments in [illegible]

Conditions of the rat experiments. The [illegible] of the Sprague Dawley strain, having [illegible] individually in cylindrical glass vessels [illegible] wood shavings. The temperature in [illegible] permitted to consume the diets *ad lib* [illegible] the exception of the first experiment, the

Table II. Amino acid composition of the wheat flour and the non-supplemented bread baked from the flour and used in the present investigation. Determination according to Moore and Stein after hydrolysis in 6N HCl for 30 h

Amino acid	Wheat flour[1] g/16 g N	Wheat bread g/16 g N
Alanine	2.78	2.52
Arginine	3.18	3.14
Aspartic Acid	4.57	4.45
Cystine	—	—
Glutamic acid	36.0	30.8
Glycine	3.40	3.14
Histidin	1.64	1.90
Leucine	6.89	6.45
Isoleucine	3.94	3.72
Lysine	1.93	2.60
Metionine	1.34	1.31
Phenylalanine	4.80	4.48
Proline	12.1	10.4
Serine	3.73	3.85
Threonine	2.30	2.57
Tryptophan	—	—
Tyrosine	2.50	2.50
Valine	4.48	4.36

Nitrogen content 1.82 %, dry weight 83.7 %
Nitrogen content 2.23 % dry weight 89.5 %

for a period of 5—7 days before they were given the various experimental diets. This was done in order to accustom the rats to bread diets.

Table I summarizes the dry weights, nitrogen contents and amounts of added L-lysine HCl (Plixer & Co, Inc.) and DL-threonine (Fluka, A. G.) for the various groups of diets used in the three different rat experiments. The figures given for L-lysine HCl and DL-threonine are approximate and represent the amounts calculated before the baking was done on the basis of the weight of air-dried bread obtained from a certain weight of wheat flour. The baking of the bread for these experiments as well as for Experiment IV (see Table III and IV) was done on separate occasions.

Results

Amino acid determinations. The amino acid compositions of the wheat flour and the non-supplemented bread are given in Table II. The values are expressed as g per 16 g of nitrogen according to practice, although this is not entirely correct for cereal products. There are only rather minor differences between the amino acid compositions of flour and bread, the more noticeable ones being the changes in glutamic acid and lysine. The increase in lysine is due to the inclusion of roller-dried skim milk and yeast in the bread.

Table III. Recovery of added L-lysine HCl from bread and diets as determined by microbiological assays. For details of the calculations see text

Exp.	Group	Experimentally determined L-lysine HCl in per cent of the dry weight of the		Calculated content of L-lysine HCl in per cent of the theoretical dry weight of the		Recovery %
		Bread	Diet	Bread	Diet	
I	D	0.45		0.50		[illegible]
	D		0.53		0.53	100
II	A	0.45		0.60		75
	A		0.45		0.54	[illegible]
	B	0.52		0.60		87
	B		0.42		0.54	78
III	C	0.41		0.48		85
	C		0.40		0.43	93
	E	0.48		0.60		[illegible]
	E		0.45		0.54	83
	F	[illegible]		0.60		83
	F		0.40		0.54	85
IV		0.54		0.60		98
			0.48		0.54	89
					Average	84.8 ± 1.7*

No corresponding r t experiment reported here
Standard error of the mean.

Table IV. Recovery of added L-threonine from bread and diets as determined by microbiological assays. For details of the calculations see text

Exp.	Group	Experimentally determined L-threonine in per cent of the dry weight of the		Calculated content of L-threonine in per cent of the theoretical dry weight of the		Recovery %
		Bread	Diet	Bread	Diet	
II	B	0.10		0.18		56
	B		0.09		0.15	60
III	F		0.06		0.135	45
IV		0.07		0.12		58
			0.07		0.11	64
					Average	56.6 ± 3.2*

No corresponding rat experiment reported here.
Standard error of the mean

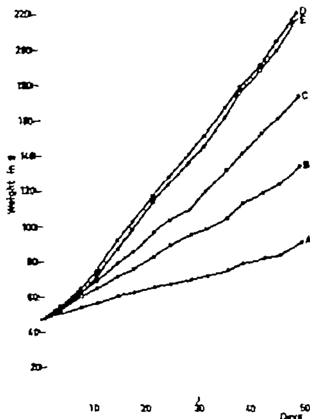


Fig. 1. Growth curves for the rats in Experiment I.

Curv. A Non-supplemented bread diet.

Curve B: Bread diet containing 0.12 % added L-lysine HCl.

Curve C Bread diet containing 0.25 % added L-lysine HCl.

Curve D Bread diet containing 0.50 % added L-lysine HCl.

Curve E Bread diet containing 0.80 % added L-lysine-HCl.

The L-lysine HCl was incorporated into the dough.

Before presenting the data on the loss of free lysine and threonine during baking as estimated by microbiological methods, it is necessary to discuss briefly how the calculations were performed that led to the figures shown in Table III and IV. Firstly it was assumed that the flour for the dough was accurately weighed and used without loss. Secondly the dry weight of the flour was assumed to be the same (86 %) on all occasions although dry weight determinations were not made in each case. Thirdly a loss of 4 % due to carbon dioxide, alcohol, etc. was calculated to take place during the fermentation, proofing and baking of the bread. Taking into account the amount of dry matter supplied by the other ingredients of the dough, one finds that 1 900 g flour should theoretically give 1 720 g of dry bread.

Since known amounts of L-lysine HCl or DL-threonine were added per unit weight of flour it is possible to express the amount of added amino acid as per cent of the theoretical dry weight of the bread. Knowing the amount of air-dried bread in the diets and the dry matter content of this bread, one

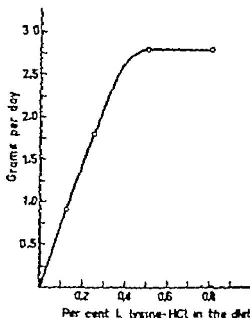


Fig. 2. Growth rate for the rats in Experiment I in g/day above that obtained with the non-supplemented bread diet as function of the amount of added L-lysine HCl expressed as per cent of the fresh weights of the diets

can also calculate the per cent of added lysine and threonine on the basis of the theoretical dry weight of the diet. Determinations of lysine, threonine and dry matter were done for both breads and diets and a recovery value was calculated as shown in the tables. This recovery value is expressed as the content found experimentally divided by the theoretical content, calculated as outlined above, and multiplied by 100.

It should be mentioned that the data in Table III and IV represent only part of all the estimations done. Only data from experiments with such levels of added lysine and threonine that were considered of practical importance (see under "Rat experiments") are presented. It should also be pointed out that the recovery as given in the tables comprises not only the loss during baking (including fermentation and proofing) but also during the drying of the bread and the hydrolysis of the samples preceding the amino acid assays.

The loss of lysine estimated by microbiological methods and expressed as just outlined is illustrated by the values in Table III. The loss of threonine is shown in Table IV. The values will be discussed further (see under "Discussion") after that the results from the rat experiments have been presented.

Rat experiments. In the first series of rat experiments, 5 different groups of rats were used, initially comprising 12 animals each. The first group received a non-supplemented bread diet and the four subsequent groups diets containing breads baked from doughs to which various amounts of L-lysine HCl had been added. The level of L-lysine HCl added corresponded to approximately 0.12, 0.25, 0.50 and 0.80 % of the fresh weights of the diets.

The growth curves are shown in Fig 1 It can be seen that the growth rate increased with increasing concentrations of lysine up to the level of 0.50 %. Above this concentration, no improvement in growth was observable. This is more easily seen from Fig 2 where the increase in growth rate in grams per day above that of the group receiving the non-supplemented bread diet is plotted against the concentration of added L-lysine HCl expressed as per cent of the fresh weights of the diets. Fig 2 shows that a nearly linear relationship exists between the growth rate and level of added lysine as long as the concentration of the L-lysine HCl is less than approximately 0.35 %. Previous experiments in which the L-lysine HCl was added after baking (ERICSON and OVERBY 1959) had demonstrated that no improvement in growth rate was obtained when the level of L-lysine HCl was increased above 0.40 % of the diet weight. This, together with the significant effect observed in the present experiment even with the lowest concentration of lysine incorporated into the dough, suggested that the inactivation or destruction of lysine during baking was comparatively small.

If the growth rates are calculated from the eighth day onwards, when the growth curves in Fig 1 are nearly linear the following values are obtained for the different groups (mean and S. E.) A 0 78 ± 0.03 B 1.67 ± 0 04 C 2.57 ± 0 06 D 3.59 ± 0 02 and E 3.57 ± 0.05

Six of the animals in each group were killed after 22 days and the remaining after 50 days. On all of the rats, determination of body fat and liver fat as well as histological examinations on the livers were carried out. The results of these observations together with those on rats from a number of other experiments — including Experiments II—IV — will be presented in a separate paper (LARSON, RUBARTH and ERICSON 1962)

In the next series of experiments various combinations of lysine and threonine were added to the dough in order to gain information on the optimum concentrations of these amino acids when they are incorporated together before baking as well as the degree of destruction or inactivation of the threonine. Five groups of rats, each comprising 10 animals, were given the diets during 36 days. The levels of L-lysine HCl and DL-threonine incorporated — expressed in per cent of the weights of the diets — are given in Table V together with the results. It is obvious that the addition of threonine to a dough already containing lysine results in a significant improvement of the nutritive value of the bread. This is in accordance with observations made previously that a combination of both lysine and threonine is superior to lysine alone when the amino acids are added to bread diets after baking (ERICSON 1960) The combination of 0.50 % L-lysine HCl and 0.30 % DL-threonine was also found to be almost as effective as 0.80 % L-lysine HCl and 0.30 % DL-threonine (Table V) A plot of gain of weight above that of the control group versus added DL-threonine indicated that the maximum effect had not been obtained at the highest level (0.30 %) tested in this series.

Table V. Composition of diets and growth rates for the five groups of rats in Experiment II. Ten rats were used per group. The amino acids were added before baking.

Group	Diet	Growth rate g/day
A	Basal + 0.50 % L-lysine HCl	3.96 ± 0.09[1]
B	As A + 0.30 % DL-threonine	6.05 ± 0.09
C	Basal + 0.80 % L-lysine HCl + 0.08 % DL-threonine	5.03 ± 0.03
D	As C but with 0.15 % DL-threonine	5.68 ± 0.06
E	As C but with 0.30 % DL-threonine	6.36 ± 0.15

[1] Standard error of the mean.

Table VI. Composition of diets and growth rates for the six groups of rats in Experiment III. Twelve rats were used per group.

Group	Diet	Growth rate g/day
A	Basal + 0.30 % L-lysine HCl added *after* baking	3.39 ± 0.10[1]
B	Basal + 0.40 % L-lysine HCl added *after* baking	4.03 ± 0.11
C	Basal + 0.40 % L-lysine HCl added *before* baking	3.75 ± 0.02
D	Basal + 0.50 % L-lysine HCl added before baking + 0.15 % DL-threonine added *after* baking	4.97 ± 0.05
E	Basal + 0.50 % L-lysine HCl added before baking + 0.25 % DL-threonine added *after* baking	5.15 ± 0.05
F	Basal + 0.50 % L-lysine HCl added before baking + 0.25 % DL-threonine added *before* baking	4.92 ± 0.04

[1] Standard error of the mean.

In the third series, a direct comparison between the effects of bread diets to which lysine or lysine plus threonine had been added before or after baking was attempted. When lysine alone was studied, the concentrations of lysine added after baking were 0.30 and 0.40 % L-lysine HCl while the concentration added before baking was set at 0.40 %, again expressed in per cent of the fresh weights of the diets. These levels were considered appropriate since they were close to the optimum conditions for supplementation of bread with lysine alone (Exp. I). The levels of DL-threonine added after baking were 0.15 and 0.25 % and the amount added before baking 0.25 % of the diet weights. In these cases, we increased the amount of L-lysine HCl added to 0.50 %, as Exp. II had indicated this to be necessary in order to obtain full effect from the threonine supplementation.

The results are shown in Table VI It can be seen that 0.40 L-lysine HCl added before baking does not give the same growth-promoting effect as 0.40 % added after baking, but that it is better than 0.30 % added after baking The data thus indicate that the inactivation of lysine during baking of wheat bread is comparatively insignificant. In the case of threonine, the picture is different. The level of DL-threonine added before baking, *viz.* 0.25 % did not quite give the same growth rate as the lowest level, *viz.* 0.15 % added after baking This suggests a rather large loss of added threonine during the baking of bread.

Discussion

There is a large number of reports on the a ailability and heat inactivation of naturally occurring lysine in a variety of food items. These studies indicate that protein-bound lysine can be made nutritionally unavailable by at least two different types of reactions (a) by reactions with other amino acids giving unnatural" peptide linkages between the -amino group of lysine and a carboxyl group of another amino acid which are resistant to enzymic di gestion and (b) by reactions between the ε-amino group of lysine and carbohydrates (or other compounds containing aldehyde groups *e. g* gossypol) which finally leads to the formation of brown, humin-like condensation prod ucts and which again makes the lysine biologically unvailable. From the first type of reaction product, the lysine can be released by acid hydrolysis. In the latter case, this is possible only at the initial stages of the interaction — if the heat damage is too severe it becomes impossibl to recover the lysine. A third possible cause of loss — the direct monomolecular destruction of the lysine — is generally not considered to be of importance in the preparation of food for consumption.

Attempts have also been made to determine the loss of free lysine during the preparation of food, in particular during the baking of wheat bread fortified with DL- or L-lysine HCl. ROSENBERG and RHODERBURG (1951) studied this problem using microbiological assay methods. They concluded that when 0.25 % L-lysine HCl was added to the flour bout 32 % was lost during the baking However in a subsequent paper (ROSENBERG and RHODERBURG 1952) the same authors used rat experiments to investigate the loss and then found that an equal response was obtained from lysine added to flour before baking or from lysine dded in corresponding amounts to the bread diet. SARRTON and KENNEDY (1957) arrived at similar conclusion in their comprehensive feeding experiments. Their results showed no difference in protein efficiency ratios between groups of rats fed diets containing bread to which the lysine had been added befor or after baking CREVER *et al.* (1951) on the other hand, performing nitrogen balance studies on human subjects, reported an almost complete loss of free DL-lysine dded to wheat bread before baking

The loss of added L-lysine HCl in baking powder biscuits has been studied with microbiological methods by CLARK *et al.* (1959) and was found to be less than 10 %.

Our own animal experiments indicate that 85—90 % of the free L-lysine HCl added to the dough is present in a biologically available form in the bread. The microbiological assays gave recovery values for breads and diets ranging from 75 to 100 % (average about 85 %). It should once again be pointed out that this figure represents the recovery of the lysine not only from the baking process but also from the drying of the bread and the hydrolysis of the samples.

Judging from the observations of SABISTON and KENNEDY (1957) and of ROSENBERG *et al.* (1951, 1958) it seems unlikely that the drying procedure used here would have caused any significant loss of added lysine. The hydrolysis and accompanying operations, on the other hand, might well give rise to some loss and thereby lower the recovery values. This possibility was tested by adding a known amount of L-lysine HCl to a bread sample and submitting the mixture to hydrolysis under the conditions described earlier. The recovery was 92 %. (Adding the same amount of L-lysine HCl to the bread after hydrolysis resulted in a 100 % recovery.) Assuming on the basis of this observation a 5—10 % loss during the hydrolysis and subsequent operations, one arrives at a recovery figure for the loss of free L-lysine HCl during baking alone of 90—95 %. This figure agrees well with that obtained from the rat experiments keeping in mind that acid hydrolysis often releases more lysine than enzymic processes.

There seems to be no data published on the loss of free added threonine during the baking of wheat bread. Although it is known that some loss of protein-bound threonine can take place during the heating of carbohydrate-rich foods, we did not expect to find the loss of free threonine, as judged by both rat experiments and microbiological estimations, to be considerably greater than that of lysine. The former type of assay indicated that only about 60 % of the DL-threonine added to the dough was biologically available in the bread. The microbiological determinations gave recovery values ranging from 45—64 % with an average value of about 57 %. The loss of threonine during the hydrolysis was found to be rather large, approximately 25—30 %. (Adding the threonine to bread after hydrolysis resulted in a 100 % recovery.) Taking these latter figures into account, one finds that the recovery of threonine from the baking and drying processes as estimated by microbiological methods would be about 75—80 %. The reason for the discrepancy between the rat assay and the microbiological assay is not known, but the data suggest that free threonine can be made biologically unavailable to higher animals by reactions with components in food to compounds from which it can however be partially released by acid hydrolysis. Further studies are necessary to clarify this point. It is conceivable that the potassium bromate present in the flour (30—40 mg/kg) was partly responsible for the loss of free threonine.

A direct comparison between the amino acid composition of the wheat flour and bread used in this investigation and literature data has not been possible as, to the author's knowledge, such determinations have not previously been made on Swedish flour and bread. However SIHLBOM (1961) has recently carried out a study on the amino acid composition of a number of varieties of Swedish wheats and also on one sample of wheat flour that contained, after extraction of lipids, 1.67 % nitrogen. The values obtained are in good agreement with those reported here, possibly with the exception of histidine leucine and serine which showed slightly higher values in the analyses of SIHLBOM.

The baking properties of the type of wheat bread investigated in this study was not significantly altered by the addition of the amount of L-lysine HCl necessary for optimum improvement of the nutritive value (about 0.40—0.45 % L-lysine HCl based on the fresh weight of the flour). Only a slightly darker crust was noticed in most cases. No changes in taste were observed but properly planned tests to detect any differences were not performed. Doubling the lysine addition, however gave a much darker and also considerably more compact loaf of bread. The addition of a few tenths of a per cent of DL-threonine to lysine-fortified bread was considered by some of us to give an undesirable flavour to the crust. Again it should be emphasized that this observation was wholly subjective.

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From the Department of Physiology, University of Göteborg, Göteborg, Sweden

The Comparative Effects of Angiotensin and Noradrenaline on Consecutive Vascular Sections

By

Björn Folkow, Börje Johansson and Stefan Mellander

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Abstract

Folkow, B., B. Johansson and S. Mellander. *The comparative effects of angiotensin and noradrenaline on consecutive vascular sections.* Acta physiol. scand. 1961. 53. 99—104. — The action of l-noradrenaline and angiotensin on the resistance and the capacitance vessels in the skin-muscle region of the cat was studied by means of a technique recently described by Mellander (1960). Intravenous and close arterial infusions of the two substances showed that the constrictor effect of angiotensin on the resistance vessels is greater than that of corresponding amounts of noradrenaline. In the capacitance section, on the other hand, the constrictor responses to noradrenaline by far exceed those to angiotensin. On the basis of these results it is suggested that noradrenaline "receptors" are to be found on the arterial as well as the venous side of the circulation, while angiotensin "receptors" are essentially confined to the smooth muscles of the precapillary vascular region.

Among the various vasoactive substances formed in the organism angiotensin is of special interest owing to its potency and its possible role in certain forms of experimental and clinical hypertension. During the last few years the chemical structure of different angiotensin compounds has been established and the preparation of synthetic analogues has greatly facilitated investigations on the pharmacology of these substances.

The present stage of knowledge concerning the cardiovascular action of angiotensin has recently been reviewed (Schachter 1960, p. 95) and will here

only be briefly summarized Angiotensin II now considered to be the final link in the renin-angiotensin system, appears to constrict powerfully the resistance vessels in all systemic vascular regions with possible exception of the coronary vessels. *The circulatory effects of angiotensin on the different 'parallel-coupled'* vascular circuits supplying the various tissues, are thus fairly well-known as regards their resistance vessels. However the action of vasoactive agents should also be considered from another point of view Each regional vascular circuit is made up of a number of functionally differentiated 'series-coupled' sections, which can be called 'Windkessel vessels 'resistance vessels with a precapillary and a postcapillary section, 'sphincter vessels exchange vessels capacitance vessels and in some tissues shunt vessels (For details see *e. g* FOLKOW 1959 or MELLANDER 1960) Not only reactions of the resistance vessels, as a whole are here of importance but also the relation of constriction, or dilatation, *between the precapillary and postcapillary resistance vascular sections, since* this ratio will affect mean hydrostatic pressure in the capillaries and hence the filtration exchange across their walls. Further the effects on the tone of the capacitance vessels are also highly important as they will markedly affect the venous return of blood to the heart.—As the phasic reactions of these functionally differentiated consecutive vascular sections can now be studied simultaneously in a skin-muscle region in the cat, by means of a technique recently described by MELLANDER (1960) it was considered of interest to utilize this method for a comparative study of the action of angiotensin and noradrenaline on resistance and capacitance vessels. — The present results have previously been briefly outlined (FOLKOW *et al* 1960)

Method

The present investigation was performed on 15 cats, anaesthetized with chloralose (50 mg/kg) and urethane (100 mg/kg) intravenously In few cases dial (Ciba, 30—40 mg/kg) or urethane alone (1,000—1,500 mg/kg) was used, but the different anaesthetics did not in principle affect the results. As indicated bove, simultaneous recording of phasic reactions in consecutive vascular sections is possible by means of the method used in these experiments. This method has been described and tested in detail by MELLANDER (1960 and is also in principle described in nother paper in the present volume (FOLKOW MELLANDER and OBERG 1961) It allows an analysis of the extent of the reactions of the pre- and postcapillary resistance vessels separately as well as the capacitance vessels. — As the direct action of the vasoactive agents upon the vessels of the hind quarters was to be analysed, the lumbar sympathetic chains were generally cut in the course of the experiment, n order to record the pharmacological effects on the vascular bed *per se* when t is deprived of all reflexogenic hanges of vasoconstrictor tone *Disturbances from endogenous adrenomedullary hormones were prevented by* extirpation of the righ suprarenal gland and denervation of the left one.

Angiotensin II (Ciba and l-noradrenaline were given as intra-venous and close arterial injections and infusions, by w y of one of the brachial veins and a suitable lumbar branch *of the aorta, respectiv*)

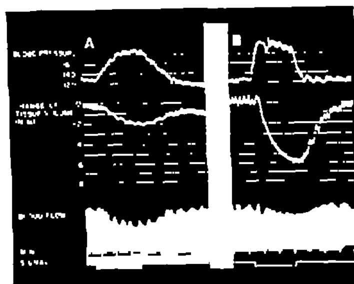


Fig. 1 Cat, chloralose-urethane.
The effects on blood pressure, 'blood volume' and blood flow of intra-venous infusion of angiotensin, 1.2 μg/kg/min (A) and l-noradrenaline, 1.2 μg/kg/min (B). Note the bigger increase of flow resistance and the smaller effect on 'blood volume' of angiotensin as compared with l-noradrenaline.

Results

Fig. 1 show the vascular responses recorded in a representative experiment, where angiotensin and l noradrenaline were given in intra-venous infusions. The upper tracing represents the arterial blood pressure, the middle one the changes in regional tissue volume and the lower one the regional blood flow, the height of the ordinates being directly proportional to the rate of blood flow.

In Fig. 1 A angiotensin was infused intra venously at a rate of 1.2 μg/kg/min, which induced an increase in blood pressure and decrease in blood flow corresponding to a change in vascular resistance from 13 to 33 relative units. Regional tissue volume was slowly and moderately decreased. The two phases in the volume curve due to active constriction of the capacitance vessels and transcapillary influx of fluid are not so clearly distinguished here. However a fall in mean capillary hydrostatic pressure can be assumed on the basis of the induced changes in vascular resistance and blood pressure. Thus, the total regional resistance has increased approximately 2.5 times the initial value and most of this increase should occur in the precapillary resistance section, since the venous side is on the whole very little affected judging by the slight volume decrease. In spite of the elevated arterial perfusion pressure, thus predominant precapillary vasoconstriction should induce a lowered capillary pressure, and hence at least part of the recorded volume change should be due to fluid reabsorption

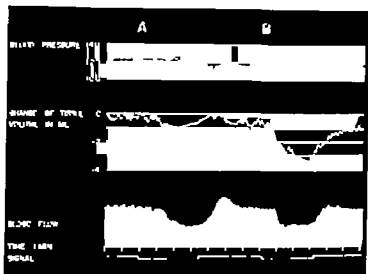


Fig 2. Cat, chloralose-urethane.
The effects on blood pressure, blood volume and blood flow of intra-arterial infusion of angiotensin, 1 4 μg/kg perfused tissue/min (A) and l-noradrenaline, 3.6 μg/kg perfused tissue/min (B). Note the bigger decrease of volume caused by l-noradrenaline, when this drug and angiotensin are given in doses that elicit approximately similar increases of flow resistance

In Fig 1 B l-noradrenaline, 1.2 μg/kg/min, was infused intra venously The elevation of arterial pressure is accompanied by a reduced regional blood flow and the vascular resistance is here increased from 13 to 22 relative units. In the plethysmographic curve the two different slope characteristics are easily distinguished the initial, rapid decrease in volume due to active constriction of capacitance vessels and the following, continuous one, due to transcapillary absorption of extravascular fluid. In this case the contribution of these two processes to the recorded volume decrease is approximately 5 and 1 5 ml respectively — Fig 1 has thus revealed some differences in the action of noradrenaline and angiotensin on resistance and capacitance vessels, which will be further discussed later on.

Similar results were obtained in Fig 2, where the two substances were administered intra arterially This figure illustrates first the effect of angiotensin (A) infused at a rate of 1 4 μg/kg tissue/min. As here angiotensin is not generally distributed, there is no definite change in arterial blood pressure, but the regional vascular resistance of the hind parts is gradually increased from 10 to 33 units. Tissue volume is, on the whole very little affected, but the small initial deflection implies a minute decrease in regional blood content. This is possibly the result of a slight active constriction in the capacitance vascular section, but a mere passive reduction in the diameter of these vessels, due to intra vascular pressure drop cannot be excluded. The vascular effects at this particular infu-

tion are not stable enough to give any clear indication of significant changes in capillary fluid exchange.

In Fig 2 B the effect of l-noradrenaline 3.6 μg/kg tissue/min, given intra arterially is demonstrated. The effect of this dose on the total regional resist ance is quantitatively equivalent to that of the smaller dose of angiotensin in Fig 2 A. The volume decrease, on the other hand, is here considerable, comprising approximately 3 ml. The two phases in the volume curve are also recognized more distinctly in this recording — Similar vascular effects were also obtained in such experiments where the doses of the two agents were lower than those here given. The effects described were easily reproduced in different experiments.

Discussion

The present experiments indicate that angiotensin induces a stronger constriction of muscular and cutaneous resistance vessels than noradrenaline, when given in equivalent doses. On the other hand the constrictor effect of noradrenaline on the capacitance vessels is much more pronounced than that of angiotensin. Thus, when the two agents were given in such amounts as to induce the same degree of constriction in the resistance vessels, the decrease in tissue volume induced by noradrenaline was up to 5 times as great as that caused by angiotensin. The difference in "active" constriction of the capacitance vessels — mainly the veins — might be even more pronounced, since other factors can also be expected to produce a volume decrease when angiotensin is given. Thus, a constriction of mainly the precapillary resistance vessels will necessarily lower the hydrostatic capillary pressure, other factors being unchanged, and hence tend to cause an extravascular fluid absorption. Further such a precapillary vasoconstriction implies under the prevailing circumstances a decrease in postcapillary transmural pressure, which will cause a passive-elastic recoil of the highly distensible venous side, mobilizing some of its blood content Lastly the constriction of the precapillary vessels will lower their blood content. Such mechanisms may to a great extent, possibly entirely be made responsible for the slight decreases in tissue volume observed during angiotensin infusion. Since the venous side of the vascular bed constitutes the main part of the capacitance section and the arterioles the most important resistance section, this investigation makes it likely that the smooth muscles of the veins, as well as those of the arterioles, are supplied with noradrenaline 'receptors' On the other hand, angiotensin 'receptors' seem to be mainly if not totally confined to the precapillary vascular region. If angiotensin was given in such concentrations as may be expected to occur in certain types of hypertensive disease — raising flow resistance some 50—100 — the effects on the venous side appeared to be negligible. If, however the substance was given in massive doses, so as to stop blood flow almost entirely in the hind parts, rela

tively marked volume decreases were observed, and thus it seems possible that there might be a small number of angiotensin "receptors" also on the venous side. It should, on the other hand, be realized that under such circumstances it is no longer a matter of mimicking a reasonable patho-physiological situation in hypertensive disease but rather a toxicologic situation. — It might be argued that angiotensin could be unevenly distributed within the vascular bed, thus reaching the arteriolar smooth muscles in higher concentrations. It should, however, be recalled that, when vasoactive substances are given by way of the blood stream, the smooth muscles of the arterioles, as well as those of the veins, are in all probability mainly reached from the tissue spaces via the capillary exchange, and the results show that noradrenaline must easily reach the venous side.

These results have thus revealed quantitative, and probably also qualitative, differences between the action of two pressor agents on various consecutive vascular sections, differences which would hardly be possible to discover only by means of conventional blood pressure and blood flow recordings. For a true physiological and pharmacological evaluation of the action of vasoactive drugs it seems important to apply a method that also allows a detailed analysis of the vascular responses in different "series-coupled" sections, since each of them has a highly differentiated function in cardiovascular homeostasis.

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From the Department of Physiology, University of Göteborg, Göteborg, Sweden

Autoregulation and Basal Tone in Consecutive Vascular Sections of the Skeletal Muscles in Reserpine-treated Cats

By

BJÖRN FOLKOW and BENGT ÖBERG

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Abstract

FOLKOW, B. and B. ÖBERG. *Autoregulation and basal tone in consecutive vascular sections of the skeletal muscles in reserpine-treated cats.* Acta physiol. scand. 1961. 53. 105—113. — An experimental analysis has been performed in reserpine-treated cats concerning the extent of basal vascular tone and the nature of smooth muscle 'autoregulation' within the consecutive sections of the vascular bed of the skeletal muscles. The experiments suggest that these events are manifestations of a truly myogenic activity, basically independent of extrinsic sources of excitatory agents, and that it is essentially concentrated to the precapillary resistance vessels. Some implications of these data with regard to the cardiovascular system as a whole are briefly discussed.

Recent studies indicate that 'basal' vascular tone — *i. e.* the average extent of vasoconstriction, still present after elimination of the vasomotor nerves, — is a matter of local mechanisms and in principle independent of bloodborne vasoactive substances (see *e. g.* LOFVING and MELLANDER 1956). The precise nature of these local mechanisms is, however, debated. A myogenic automaticity of the contractile elements, to some extent facilitated by the distending effect of the transmural pressure, appears to be the most reasonable explanation of the basal vascular tone. To this should be added the vascular sensitivity to any variation of smooth muscle environment, whether this is caused by a pressure-induced change in blood flow, by a shift of tissue metabolism or by a changed blood composition. — Vascular circuits, exhibiting a more pronounced

basal tone, which is modified by the mentioned factors, will also exhibit an often considerable 'autoregulation' of blood flow. By autoregulation is implied the locally induced adaptations of vascular tone, which for instance, tend to keep blood flow fairly constant independent of changes in perfusion pressure. — Some aspects of these problems are more fully discussed elsewhere (Folkow and Löfving 1956, Folkow 1960).

It is, however, not yet disproved beyond doubt that locally released constrictor agents and regional reflex arcs might after all constitute the initiating mechanisms. Several authors still claim the existence of independent nerve plexa in the vascular walls, and reactions like the constrictor responses to venous distension have been ascribed to a local 'veni vasomotor reflex' (Gaskell and Burton 1953). Further, there is now good evidence of scattered chromaffin cells along the vessels (*e. g.* Adams-Ray et al. 1958), and such cells might act as local releasers of constrictor agents, such as catechol amines, thus establishing a tonic vascular contraction. — Such a concept implies, however, a fundamentally different mechanism as compared to the muscle automaticity view, as the initiation of basal vascular tone should then not originate within the contractile elements themselves, but be a matter of extrinsic though local stimuli. In case this latter view should be correct, basal tone and autoregulation could be expected to be largely eliminated in reserpine treated animals, as it is known that this drug is able to deplete the organism of catechol amines and related smooth muscle stimulating substances.

Experiments have therefore been performed on reserpine-treated cats in order to evaluate the extent of basal vascular tone and blood flow autoregulation in skeletal muscles. A technique has been utilized, which allows a simultaneous recording of the responses of the pre- and postcapillary resistance vessels and the capacitance vessels, making it possible to find out whether basal tone and autoregulation of the vascular smooth muscles is of similar degree in all parts of a vascular circuit or essentially confined to some of its consecutive sections. Such a possibility is of some principal interest as it provides information concerning the extent of functional differentiation inbetween different parts of a vascular circuit with respect to their contractile elements.

Method

Medium-sized cats were used, which during four days prior to the experiment had been treated with reserpine (Serpasil®) given intramuscularly in daily doses of 2 mg per kg. The animals were anesthetized with a combination of chloralose, 40—60 mg per kg, and urethane, 100—150 mg per kg. — The technique utilized for separate recording of the reactions of the pre- and postcapillary resistance vessels and the capacitance vessels, has previously been described and analysed in detail (Mellander 1960) and is outlined in principle in another paper in the present volume (Folkow, Mellander and Öberg 1961). For the present purpose it should only be added that the venous outflow pressure from the hind parts could be set to any wished level, and further also the filling pressure of the heart could be easily adjusted. In these animals, entirely deprived

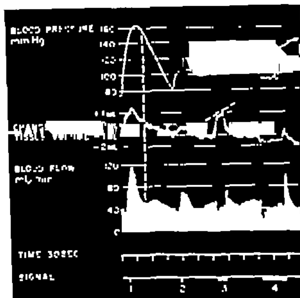


Fig 1 Reserpine-treated cat, 3.4 kg Chloralose-urethane. The figure illustrates the effects on blood flow and blood volume when arterial blood pressure is temporarily raised 90 mm Hg (1) 25 mm Hg (2), when venous pressure is raised 10 mm Hg (3) and when arterial blood pressure is lowered 40 mm Hg (4)

of functioning vasoconstrictor and accelerans nerves and with acutely cut vagal nerves, all cardiovascular reflex adjustments were eliminated. Therefore, even minor shifts of venous return from the funnel to the heart markedly affected its stroke volume, and hence cardiac output, making it easy to induce even drastic shifts in arterial blood pressure simply by changing the inflow rate from the funnel. Alternatively careful adjustments of this inflow made it possible to keep the arterial pressure fairly constant, if so wanted.

In order to exclude the skin blood flow in the hind limbs as much as possible tight ligatures were placed at the proximal ends of the paws and the tail. Generally 80—85 per cent of the studied tissue volume was found to be made up by skeletal muscles. In few cases both hind limbs were also cautiously skinned. To maintain steady state with regard to ventilation, constant artifical respiration was given to such an extent as to barely suppress the spontaneous ventilation of the anesthetized animal.

With the method used the hind parts constituted a muscle vascular bed, entirely deprive of all adrenergic nervous connections, but normally perfused from the upper half of the animal, which merely acted as physiological blood pump and oxygenator. Both the arterial inflow pressure and the venous outflow pressure could be adjusted to any wished level, allowing an analysis of the influence of arterial and venous pressure changes on both flow and regional blood volume and, in certain circumstances, also on transcapillary filtration exchange.

Results

The vascular reactions to different types of pressure changes, which with great regularity could be repeated, are best presented by describing in detail a characteristic experiment (Fig 1) At 1 and on smaller scale at 2 in the figure, a steep and marked increase of arterial pressure is induced and its effect on blood flow and blood volume can be followed. It is clear from the figure that both

flow and also volume, increases in rough proportion to the pressure rise, to start with. However while the arterial pressure rise is still at its top level both blood flow and volume increases fade away rapidly and return almost to their initial values. Obviously a pronounced vasoconstriction is initiated after a brief latency as a response to the pressure rise and, when fully established this vascular autoregulation is almost able to normalize both flow and volume, in spite of the fact that the arterial pressure is about doubled in 1 — Such a pronounced arterial pressure rise would, if vascular tone had remained the same, have raised also mean capillary and venous pressures considerably causing a lasting pronounced tissue volume increase, due to both an outward filtration and venous distension. No such phenomenon takes place, however On the contrary the initial volume increase vanishes as rapidly as does the flow increase. This fact suggests that the marked reactive increase of flow resistance takes place mainly within the precapillary resistance section, which has the hemodynamic consequence that the arterial pressure rise is no longer transmitted to the capillary and venous sections. It is, however so far still possible that some increase of postcapillary flow resistance takes place, but '3 in Fig 1 makes it clear that a pressure increase within the postcapillary venous section of the vascular bed will not cause any significant increase of smooth muscle tone in this section. — Arterial blood pressure is here kept fairly constant, but venous outflow pressure is instead suddenly increased some 10 mm Hg, simply by raising the level of the Gaddum recorder 13—14 cm. The vascular response to this manoeuvre is an immediate and rather marked volume increase, obviously due to venous distension, and this pooling of blood reduces, of course, also the venous outflow for a few seconds. However this rapid phase of venous distension is soon completed, and is then followed by a much slower though very constant volume increase It is clear from earlier analyses of such phenomena (see MELLANDER 1960) that this second slope of volume increase is a matter of outward capillary filtration and this process keeps on at a surprisingly steady rate also when the period of raised venous pressure is very long It should here especially be observed that raising the venous pressure does not induce any significant reactive tone changes within the venous section the passive distension remains unhampered and so does the rate of outward filtration, which should not have been the case if reactive shifts in venous tone had occurred. — From '2' and onwards in Fig 1 the arterial pressure is again allowed to rise, but now only gradually It is seen from the figure that both blood flow and volume are then capable to adapt themselves continuously so as to counteract the influence of the rising arterial pressure head without any noticeable time lag — In 4 the opposite of 1 is induced Arterial pressure is abruptly decreased by a partial occlusion of the abdominal aorta and kept at the lowered level for a short period of time It is seen from the flow and volume tracings that the immediate effect of the arterial pressure drop is a decrease of both flow and volume However after a latency of only some 5—10 seconds they again increase slowly

in spite of a constant arterial and venous pressure, indicating a progressive vascular relaxation, presumably also here affecting mainly the precapillary resistance vessels. This relaxation is drastically unmasked when arterial pressure is suddenly restituted, leading to a marked, but brief reactive hyperaemia and also to a volume increase.

The potency of these 'autoregulation' phenomena in reserpine treated cats are, as seen, remarkable both concerning extent and rate: the extent of autoregulation was in fact often so striking that it, if anything, was superior to that seen in sensitively reacting vascular beds of the acutely denervated cat's limb. These experiments also made it clear that basal vascular tone is essentially of normal range in reserpine-treated animals, which is obvious from the flow figures of 6—8 ml/min/100 ml of tissue for resting denervated skeletal muscles, which were recorded as long as arterial pressure was kept within its normal range. It was also clear from the fact that intraarterial injections of big doses of acetylcholine increased blood flow some five times. As a contrast to the precapillary resistance vessels, the venous side of the circulation, constituting the postcapillary resistance vessels and the main part of the capacitance vessels, had, —besides its evidently poor or absent autoregulation, — a very low basal tone. This was obvious from the fact that supramaximal amounts of intraarterially injected acetylcholine which induced often fivefold increases of blood flow calculated to correspond to about 40 % increase of internal radius of the resistance vessels, only increased the regional blood volume roughly 15—20 per cent, as calculated from MELLANDER's measurements of the blood volume in the hind part (1960). This corresponds to about 10 % increase of internal radius of the capacitance vessels. Further, much of the blood volume increase must have been due to a passive distension of the veins and to the unavoidable increase of blood content within the dilated precapillary resistance vessels. The lack of further volume increases could not be due to the fact that the dilator drug did not reach the venous side or lacked the power to relax the veins, because if venous tone had been artificially raised on beforehand by noradrenaline infusion, acetylcholine was able to induce very drastic increases of regional blood volume. — The fact that the present animals really were more or less completely depleted of catechol amines was proved by stimulations of the abdominal sympathetic trunks after that the symphatetic dilator fibres had been blocked by atropine. Even maximal excitations at highest physiological rates, which normally increases flow resistance some 7—8 times and decreases the regional blood volume about 30 per cent, — about 6—8 ml in a normal size cat (MELLANDER 1960) — had only a very minute or no effect on flow and volume.

Thus the experimental data, so far described, make it clear that catechol-depleted animals exhibit a pronounced basal tone and autoregulation, but that these manifestations of inherent smooth muscle activity were essentially restricted to the precapillary resistance vessels. Whether the autoregulation of the precapillary resistance vessels is only a matter of alternations in smooth muscle

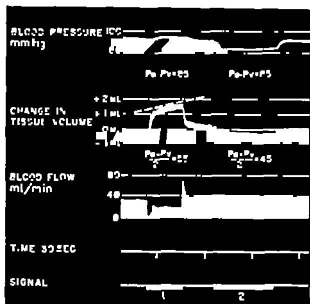


Fig 2. Reserpine-treated cat, 3.0 kg Chloralose-urethane. 'P in the figure denotes arterial inflow pressure P venous outflow pressure of the isolated hind part —The figure illustrates the effects on blood flow and 'blood olume when mean intravascular pressure $\frac{(Pa + Pv)}{2}$ is raised (1) and lowered (2) hile the perfusion pressure (Pa—Pv) is kept the same.

environment, due to the flow changes enforced upon the tissue by the primary shifts in perfusion pressure or whether also changes n transmural pressure as such contributes, is illustrated in Fig 2 In 1 and '2 of this figure the pressure head is slightly lowered to reach very nearly the same level, 85 mm Hg in the two cases. However in 1 the pressure head is reduced by raising the venous pressure about 10 mm Hg, which implies a *raised* mean transmural pressure in '2 the pressure head is reduced by lowering the arterial pressure, and hence also mean transmural pressure is decreased. It is easily calculated that the mean intravascular pressure $\frac{(Pa + Pv)}{2}$ is about 55 and 45 mm Hg, respectively in the two cases — If it is assumed as a reasonable figure that roughly 80 per cent of the flow resistance is made up by the precapillary vascular section and that tissue pressure is low and uniform, it can be deduced that the average transmural distending pressure also within the precapillary resistance vessels is some 20 per cent higher in 1 than in 2 In spite of the fact that the pressure head is the same and the chances of passive-elastic vascular distension are greater in 1 flow resistance is, in fact, almost 30 per cent *higher* n alternative 1 where the transmural pressure is raised Local 'veni-vasomotor reflexes cannot possibly have been operating in these animals, depleted of the constrictor fibre transmitter Further had the chemical environment of the smooth muscles been alone responsible of autoregulation, the blood flow would, if any thing have been slightly bigger in 1 than n '2 Finally ha ng the animal

to breath a gas-mixture containing 6 % oxygen, implying a lowering of arterial oxygen saturation to about 50—55 %, this rather drastic change in blood composition and hence in vascular smooth muscle environment, was as a rule followed by comparatively small shifts in vascular tone in the present circumstances, and these shifts were not observed until after a considerable latency. These circumstances suggest that changes in the chemical environment of the vascular smooth muscles do not form the only, possibly not even the major mechanism responsible of autoregulation. The distension of the contractile elements in the vascular walls, offered by the transmural pressure, appears to be of considerable importance in such vessels which exhibit a more pronounced basal tone.

Discussion

The present experiments make it highly unlikely that any local nerve plexa or cell structures, producing vasoconstrictor agents, should be the background of basal vascular tone and autoregulation of flow in skeletal muscles. In fact, the reserpine treatment appears to have, if anything, potentiated these inherent reactions of the vascular contractile elements, presumably because reserpine treatment implies a 'pharmacological denervation' which is known to give rise to a sensitization of the effector cells to a variety of stimuli (EMMELIN and MUREN 1951). The experiments thus strengthen the view that both basal vascular tone and autoregulation of flow are manifestations of an inherent, myogenic activity. It appears likely that this is the case also in other vascular regions exhibiting autoregulation of flow, e.g. the kidneys.

It can also be concluded from the present experiments that the distension, offered by the blood pressure, acts as a facilitating influence on the myogenic activity of the vessels, presumably by increasing the *rate* of spontaneous discharge of the individual muscle cell as has been observed for myogenically active intestinal smooth muscles (BÜLBRING 1955). If the frequency of the spontaneous contractions is raised, the time fraction occupied by the relaxation phase can be expected to be proportionally shortened, and the integrated result of this would be a somewhat raised vascular tone. — Obviously the influence of distension is only significant in vascular sections, which really exhibit a pronounced basal tone, i.e. in the precapillary resistance vessels. Where myogenic automaticity is negligible or absent, there is nothing that could be facilitated by distension.

It is quite obvious, however, that also shifts in the chemical environment of the vascular smooth muscle cells profoundly affect their tone. In some situations the influence of such changes will cooperate with the effects of shifts in transmural pressure, as in the establishment of autoregulation of flow. In other circumstances the two mentioned factors will counteract each others, for instance when blood flow is decreased by way of a raised venous pressure, or where blood pressure and tissue activity are concomitantly increased. What

the net effect on vascular tone will be in such situations may vary from one tissue to the other It can be expected to depend on the relative sensitivity of the regional smooth muscles — which is not necessarily the same in all tissues, — to the contrary-directed shifts in transmural pressure and chemical environment. Thus, even if only these two mechanisms out of the manifold that affect vascular tone are considered, one deals in fact with a highly complex integration where the balance may vary not only inbetween the different vascular circuits, but even within one and same circuit from time to time. See also the recent study by STAINSBY and RENKIN (1961) where autoregulation of muscle blood flow is discussed from a viewpoint based on a somewhat different experimental approach. — Too little is still known about the fundamental characteristics of the contractile elements of the small blood vessels to justify a more detailed discussion of these problems at present. It should be emphasized, however that in looking at the control of blood flow in relation to tissue metabolism, nervous control etc. in the intact organism, the combined action of distension and metabolic influences on the vascular smooth muscles should constantly be kept in mind too often only nervous and hormonal excitatory factors are considered

It is of interest that the pronounced basal tone and autoregul tion are essentially manifestations of only the precapillary resistance vessels from the point of view that this implies a clearcut smooth muscle differentiation inbetween the various consecutive sections of the vascular bed Among other things such a mechanism tends to maintain mean capillary pressure, and hence filtration exchange, more constant in vascular regions exposed to e g changes in hydrostatic pressure. When shifting from a recumbent to an erect position the transmural capillary pressure in the legs would be much increased with a consequent rapid edema formation if no compensatory reactions take place. A strictly locally controlled adjustment, like the mentioned myogenic autoregulation of tone in the precapillary resistance vessels, will here add to other compensation mechanisms that tend to counteract the shifts in filtration exchange. It will also automatically compensate for such regional flow increases that would have occurred by way of the distension of the precapillary resistance vessels when the transmural pressure is increased. Lastly the present findings may explain why sympathectomized subjects, being not too much disturbed in their regulation of regional and total flow resistance, practically always are much troubled by a poor control of the veins This is obvious by the prompt pooling of blood on the venous side by even slight increases of hydrostatic pressure in such subjects, which consequently affects the venous return to the heart. The present experiments suggest that both the basal tone and the power of autoregulatory adjustments are insignificant on the venous side, when taken as a unit. Obviously this most important section of the cardiovascular system is in a poor state what concerns its compensatory adjustments once its extrinsic neurogenic control is eliminated

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From the Department of Internal Medicine, University of Lund and the Wenner-Gren Cardiovascular Research Laboratory Norrtull Sjukhus, Stockholm, Sweden

Measurement of the Oxygen Diffusion Coefficient in the Subcutis of Man

By

FRANCO CALIGARA and GÖSTA ROOTH

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Abstract

CALIGARA, F and G. ROOTH. *Measurement of the oxygen diffusion coefficient in the subcutis of man.* Acta physiol. scand. 1961 *53* 114—127 — The subcutaneous tissue is considered ball which is surrounded by a network of capillaries. By applying general equations for the diffusion of gases series of equations are evolved. These equations show the importance of the different factors which influence the oxygen supply to the subcutaneous tissue. Some preliminary measurements are done in order to show the validity of these equations, and in order to show how they may be used most conveniently The increase in tissue oxygen tension when subject is switched over from breathing air to breathing pure oxygen is measured with a covered Pt electrode. From the resulting curve the oxygen diffusion coefficient is calculated. If the oxygen consumption of the tissue and the intercapillary distance is known the capillary oxygen tension and the blood flow may also be calculated. The results obtained are of the expected order of magnitude.

MONTGOMERY and HORWITZ (1950) studied how the subcutaneous oxygen tension changes during inhalation of oxygen. They measured the oxygen tension (p_{O_2}) with a platinum electrode connected to a suitable circu t and recording device. This technique has been used under various experimental and clinical conditions, see MONTGOMERY (1957)

It could also for instance be a suitable technique for studying the increase of the tissue oxygen tension p_T when a newborn infant begins to breath but before

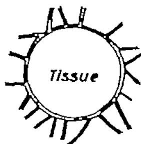


Fig. 1 The model of the oxygen supply to the tissue.

starting any series of measurements we wanted to try to evaluate the various factors that might affect the measurements. In a repetition of Montgomery experiments, a subject was given pure oxygen to breathe. The subcutaneous tissue oxygen pressure increased to a maximum and decreased again to the initial level when the oxygen was withheld. It was observed that the two branches of the curves could not be superimposed. Now it is known that an increase in arterial oxygen tension p produces contraction in the vessels of the eyes, Cusick et al. (1940) brain, Thiel (1927) Cobb and Fremont-Smith (1931) and kidneys, Fishman et al. (1951) and decrease in oxygen tension produces a dilatation. The skin, like the fundus oculi becomes pale, when a new born infant is given pure oxygen. This vasomotor reaction reduces the speed of the increase in tissue oxygen tension, and it also increases the speed with which the same tension is reduced when oxygen is withdrawn. These were both phenomena which could be observed in our curves. An interpretation of the curves of the tissue oxygen tension should therefore give a mathematical expression of the oxygen supply to the tissues and of the various factors involved. In order to achieve this, preliminary studies indicated that the arterial oxygen tension (p_a) must also be known. To show the uses of the equations evolved, some preliminary experiments have been performed and their results have been used mainly to demonstrate how to apply the equations to practical data. Thus the oxygen diffusion coefficient, which has so far only been determined in excised pieces of tissue, is experimentally determined in vivo. Also, indicative figures are obtained for the mean capillary oxygen tension, the blood flow per volume of tissue and oxygen vasomotor reaction. Although the two latter entities may be obtained by quite different measurements, it may be of value to draw as much information as possible from the arterial and tissue oxygen tensions changes.

I. Mathematical Treatment

From the tissue oxygen pressure (p_T) curve, only the oxygen diffusion coefficient can be determined. But, as will be shown, it is possible, with the use of established formulas for diffusion, to determine the factors governing the tissue p_{O_2}, the p_{O_2} of the capillaries and the blood flow if the p_T and p_a are determined at the same time.

TABLE 1

Symbols

p_T	Oxygen tissue pressure.
p_{T1} or $p_{T(t-0)}$	Initial oxygen tissue pressure.
p_c	Capillary oxygen pressure.
$\bar{p}_c$	Mean capillary oxygen pressure.
	Radius of th sphere.
	Distance from the center of the sphere to the point of measurement.
D	Oxygen diffusion coefficient.
t	Tim
m	Reaction rate of the process consuming oxygen.
α	Solubility of oxygen in the tissue.
γ	Solubility of oxygen in plasma.
β	Solubility of oxygen in whol blood.
Δp_m	Pressure drop needed for the metabolic requirement of the tissue
Δp_{cf}	Final increase in capillary and tissue pressure.
p	Arterial oxygen pressure.
P	Final increase in the arterial oxygen pressure.
P	Initial arterial oxygen pressure.
P	Conventional zero level for capillary oxygen pressure.
$p_{(\ \ c)}$	Difference between arterial and capillary oxygen pressure.
Q_1	Amount of oxygen consumed by the tissue metabolism.
Q_2	Amount of oxygen required to increase the tissue oxygen pressure.
F	Blood flow
$OVMR$	Oxygen vasomotor reaction.

The Model

Because of the abundance of capillaries in the subcutaneous tissue we have assumed a model consisting of a net-work of capillaries forming sphere and containing tissue within it. The capillaries are equivalent to the white coating of golf ball as it were, and the tissue corresponds to the substance inside. Thus the oxygen diffuses from the surface of a sphere to the interior The model is represented in Fig. 1 The oxygen tension in the capillaries (p_c) is of course different at various distances from the artery but w will only consider the mean capillary pressure ($\bar{p}_c$) as being uniform around the sphere.

The traditional model used for studying the diffusion of oxygen from the vessels into the tissues is Krogh cylinder (1919) For the following reasons we have decided to use the golf-ball model

1 The anatomical distribution of the capillaries in the subcutaneous tissue agrees better with our model than with the Krogh-cylinder

2 The Krogh-model considers only one capillary and its surrounding cylinder and disregards the influences of crossing vessels.

3 Even if the Krogh-model is extended to take several cylinders into account (Thews 1953) it is assumed that they are of an equal distance from arteries.

4 The Krogh-model assumes that the capillaries are rectilinear

The mathematical treatment will of course be different if a measurement of tissue p_{O_2} is made t one geometrical point of the sphere or if the measurement itself gives mean value over a surface which is large in relation to the size of the sphere W will begin by treating the electrode as an ideal point.

The ideal pointlike electrode

The symbols used are summarized in Table 1

If, for the moment, the oxygen consumption of the tissue is disregarded, we may use Crank formula (1956) which gives the pressure at a distance from the center of the sphere at the time t,

$$\frac{p_{T(r,t)} - p_{TI}}{p_s - p_T} = 1 + \frac{\;}{\pi} \sum^{\infty} \frac{(-1)}{\;} \sin\left(\frac{n\pi}{\;}\right) e^{-Dn^2 \; t} \qquad (1)$$

where

$p_{T(r,t)}$ = p_{O_2} in the tissue t the point at the time t.

p_T = the uniform initial p_{O_2} in the tissue independent of t and (For other symbols see Table I)

When a steady-state is reached, that is at $t = \infty$ the exponential part of (1) equals zero and the equation is reduced to

$$\frac{p_{T(r)} - p_T}{p_s - p_T} = 1 \qquad (1\;)$$

This equation is only fulfilled if $p_{T(r)} = p_s$.

It follows then from study of this equation that, if there is no oxygen consumption, in steady-state, the p_{O_2} inside the sphere is the same everywhere and equals the pressure in the capillaries.

As the tissue through which the oxygen diffuses, consumes oxygen, it is necessary to introduce factor for the metabolic oxygen consumption Roughton (1952) studied the problem of the diffusion of a gas into a sphere where t the same time the gas is undergoing zero order reaction by which it is consumed. Using the same symbols as before his equation is

$$p_{T(r,t)} = p_s - \frac{m(a^2 - r^2)}{6D} + \frac{2}{\pi} \sum_{1}^{\infty} \frac{(-1)^n}{\;} \left[\frac{ma^2}{Dn^2\pi^2} - (p_s - p_{TI})\right] \sin\left(\frac{\pi r}{\;}\right) e^{-Dn^2 \; \pi^2 t/a^2} \qquad (2)$$

where

m = the reaction rate of the process consuming oxygen

α = the solubility of oxygen in the tissue.

This equation is valid if the amount of oxygen consumed by the tissue is independent of the p_{O_2} (zero order reaction) Chance (1957) has shown that this is the case over wide range of pressure, and it is assumed in the present work that m is independent of p_{O_2}.

At steady state ($t = \infty$) the exponential part of (2) equals zero and the equation is reduced to

$$p_{T(r)} = p_s - \frac{m(a^2 - r^2)}{6D} \qquad (2\;)$$

Equation (2) is symmetrical with respect to the center of the sphere which means that the p_{O_2} is constan over any concentric spherical surface within the sphere. It may also

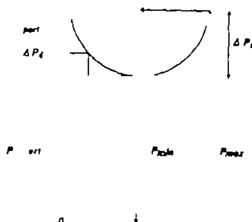


Fig. 2 The meniscus represents the p_{O_2} along a diameter. This p_{O_2} goes from minimum p_{min} at the center to maximum p_{max} at the periphery. The p_T which is measured may assume any value within these limits depending upon where inside the sphere the point of measurement happens to be placed.

be seen from (2 a) that when r decreases p_T also decreases and that p_T has a minimum at the center of the sphere, where r is zero and a maximum at the surface where $r = a$.

From (2 a) it is evident that at the periphery of the sphere, where $r = a$ also $p_T = p_c$ i.e. the tissue pressure is the same as the mean capillary pressure. The difference between the highest p_T (p_{max}) at the periphery of the sphere and the lowest p_T (p_{min}) in the centre of the sphere ($p_{max} - p_{min}$) we call Δp_t. The shape of the meniscus in fig 2 and the value of Δp_t are given by the second term of the right-hand member of the equation (2 a) and are therefore not affected by the absolute value of the p_{O_2}. Δp_t represents the pressure required to furnish all and only the amount of oxygen necessary to the tissue for its own metabolic requirements and it depends upon the reaction rate (m) the diffusion coefficient (D) and a geometrical factor which in our case is the radius a.

It follows that changes in the capillary pressure will neither affect the value of Δp_t nor the shape of the meniscus, unless the mean capillary pressure itself becomes lower than Δp_t. For physiological reasons it may be assumed that p_c never becomes lower than Δp_t.

In order to calculate the various factors governing the transportation of oxygen to the tissue it is convenient to divide the tissue pressure and the capillary pressure into two reciprocally corresponding parts, part A and part B.

Part A in the capillaries is Δp_t and in the tissue may assume any value between zero and Δp_t depending upon the distance from the center of the sphere. As has been stated Δp_t represents the pressure needed to furnish the tissues with their metabolic requirements. Once the tissue obtains the oxygen it needs for its metabolism, part A is in steady state and equation (2) therefore is simplified to equation (2 a). On further examination of equation (2 a) and Fig 2, it will be remembered that $p_c = p_{max} = p_{T(r=a)}$ and $p_{min} = p_{T(r=0)}$. Introducing $r = O$ and $r = a$ into (2 a) and subtracting we get

$$\Delta p_t = p_{max} - p_{min} = \frac{m a^2}{6 D} \qquad (3)$$

Part B is that part which is not influenced by the oxygen consumption of the tissue and is governed by equation (1) or (1 a). This part is independent of r and t in a steady state but dependent on them in a dynamic state. The corresponding part of the capillary pressure is ($p_c - \Delta p_t$) as Δp_t was part A governed by the oxygen consumption. An

change in the capillary pressure is reflected only in this part B of the tissue pressure. From (1 a) it will be seen that, in steady state, part B of the tissue pressure is the same as the corresponding capillary part and is *therefore equal to* $(p_c - \Delta p_c)$

The information available in steady state is not adequate to solve the equations, whereas in dynamic condition sufficient amount of data may be obtained. Such condition would occur if the p increased as during the breathing of oxygen. Thus, in order to calculate the capillary pressure we must find mathematical relationship between the tissue pressure and the capillary pressure in dynamic conditions.

The increase in the arterial oxygen pressure when pure oxygen is breathed must be governed by *simple exponential function of time as it depends upon the washing out* of nitrogen from the alveoli.

$$p_a(t) = P\ (1 - e^{-\delta t}) + P \tag{4}$$

where

P = difference between the initial and the final pressure.
P = initial pressure.
δ = constant.

This equation we have found experimentally

The constants P_1, P and δ may be determined from measurement of the p_a during oxygen breathing. When the pressure in the arterial blood has an exponential form, it seems reasonable to expect similar form for the oxygen pressure in the capillaries. It is assumed that the oxygen tension in the capillaries is governed by an equation of the same type as (4) but with different constants.

The equation is

$$p_c(t) = \Delta p_f\,(1 - e^{-\beta t}) + P \tag{5}$$

where

Δp_f = the difference between the initial and the final pressure which, as is shown in Fig 3, is equal to the total increase in the tissue pressure and may therefore be determined experimentally
β = constant.
P = another constant representing the initial pressure.

If the p_{O_2} in the capillaries is governed by equation (5) then p_{O_2} inside the tissue sphere is governed by another equation, CRANK (1956)

$$p_T(r,t) = \Delta p_f - \frac{\Delta P_f}{r}\, a\, \frac{\sin\sqrt{\frac{\beta r^2}{D}}}{\sin\sqrt{\frac{\beta a^2}{D}}} - \frac{\Delta p_f\ 2\beta a^2}{r D}\sum_{1}^{\infty}(-1)^n\ \frac{e^{-Dn^2\pi^2 t/a^2}}{n(n^2\pi^2 - \beta a^2/D)}\sin\left(\frac{n\pi r}{a}\right) \tag{6}$$

(6) does not give the absolute pressure, but only the increase from the initial steady state. The initial pressure corresponding to P in (5) must be added to (6) in order to represent the total tissue pressure. p_T in (6) therefore, has slightly different meaning than in the other equations, but it might be confusing to introduce new symbol for the tissue pressure in this equation.

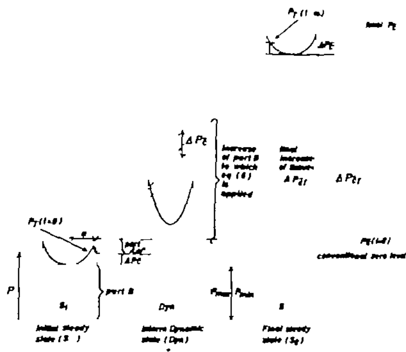


Fig 3 is schematic representation of the tissue pressure changes during oxygen inhalation.

There is to the left an initial steady state (S_1) and to the right final steady state (S_2) in between which there is continuous series of dynamic states, one of which is represented as Dyn.

Because of th steady state in S_1 and S_2 the shape of the menisci are equal as shown in the Fig It then follows from geometrical considerations that Δp_{Tf} = total increase in the tissue pressure. I the intermediat dynamic state the shape of the meniscus is altered because it is formed by the original shape of the meniscus and superimposed meniscus due to the flow into the sphere of oxygen needed for an increase in tissue pressure.

Equation (6) is applied to the part indicated.

In order to get p_a part A and p_{mean} must be added to the pressure from equation (6) p_{mean} is the initial Part B. Or in other word The final mean capillary pressure is btained by adding he observed increase in tissue pressure to the initial tissue pressure which consists of part A + part B.

P in (5) is used as conventional zero level and is the p_a of the initial steady state.

Equation (6) shows that p_T (,t) is made up from a summation of exponential terms.

Before we can apply (6) t should be shown that an experimental p_T curve fulfills such conditions, and in ttempts to rectify the p_T curv we found this to be the case.

In order to calculat p_a β must be known and this is accomplished by solving (6). If for the sake of convenience we writ (6) as $h(t)$ then for $t = t$ we get

$$p_T(t \quad) - p_T(-\infty) = h(t) \tag{7}$$

(7) contains 3 unknowns, namely D and β If we establish a set of 3 equations (7) for different values of t the system may be solved and the constants determined.

In order to solve (5) and obtain p_a only P remains.

As mentioned P is $p_{(t\to\infty)}$. From (2) it follows that

$$p_{(t\to\infty)} = p_{T(t\to\infty)} + \frac{m(a^2 - r^2)}{6D} = P \tag{8}$$

This can only be used if m and a are known from independent measurements whereas and D as just stated, are obtained from (7). P is then calculated and from this p_i in (5)

The blood flow may be calculated from the amount of oxygen which enters the tissue. I order to obtain this several steps are needed.

The difference between the brachial artery p_{O_2} and the p_{O_2} in the capillaries $(p_{(a-t)})$ is then given by (4) subtracted from (5) that is by

$$p_{(a\ \ t_{(t)})} = P\,(1 - {}^{-\beta t}) + P - \Delta p_{if}(1 - e^{-\beta t}) - P \tag{9}$$

The amount of oxygen that enters the surface of the tissue sphere per unit of time is given by two terms equivalent to parts A and B one corresponding to the oxygen consumption of the tissue, which is constant and given by

$$\frac{\partial Q_1}{\partial t} = 4\pi m a^3/ \tag{10}$$

and the other corresponding to the oxygen needed for increasing the pressure inside the sphere. This is calculated by multiplying (6) by α, the solubility of oxygen in the tissue, which gives the oxygen concentration in function of t and If we multiply this by $4\pi r^2$ and integrate with respect to within the limits and a, we obtain the amount of oxygen present in the sphere in function of time. Then by derivating with respect to t, w obtain the amount of oxygen entering the surface of the sphere per unit of time in func tion of time. The result of all these operations is

$$\frac{\partial Q_2}{\partial t} = 4\alpha\pi\ \ \Delta p_{if} D e^{-\beta}\left[1 - \sqrt{\frac{\beta a^2}{D}}\ \cot\sqrt{\frac{\beta a^2}{D}}\right] - \tag{11}$$

$$- 8\alpha a^3 \Delta p_{if}\beta \sum_{n}^{\infty} \frac{\pi}{(n^2\pi^2 - \beta a^2/D)} e^{-Dn^2\ \ \pi/a^2}$$

Calling $Q\,(t)$ the total amount of oxygen entering the sphere per unit of time

$$\frac{\partial Q}{\partial t} = \frac{\partial Q_1}{\partial t} + \frac{\partial Q_2}{\partial t} \tag{12}$$

Calling $F\,(t)$ the blood flow through the capillaries per unit of time and γ the solubility of oxygen in the blood w have

$$F_{(t)}\,\gamma\, p_{(a-t)(t)} = \frac{\partial Q}{\partial t} \tag{13}$$

and

$$F_{(t)} = \frac{\partial Q}{\partial t}\ \gamma\, p_{(a-t)(t)} \tag{14}$$

When using equations (13) and (14) it must be remembered that γ may be considered constant only in limited parts of the dissociation curve

Calling F_{air} the blood flow when breathing air we may now define the oxygen vaso motor reaction $OVMR$ as

$$OVMR = \frac{F_{air}}{F_{(t)}} \tag{15}$$

The actual electrode

The pointlike electrode only represents an ideal case which cannot at present be realized because the electrodes availabl have a size which has the same order of magnitude as the tissue sphere assumed as a model. Therefore, the treatment described above has to be modified in order to fit the experimental circumstances. If the active surface of the electrode is of the same order of magnitude as the maximum section of the sphere the pressure measured is not the pressure t on point of the sphere but an verage pressure over all the sphere.

The tissue pressure read off with the electrode is then not the pressure given by (7) but is th pressure given by

$$\bar{p}_T(t) = \frac{1}{a} \int (7)\, dr \tag{7 }$$

Since the direct integration expressed in (7 a) is not obtainable in finite terms the following procedure is adopted Multiplying (7) by α (oxygen solubility in the tissue) obtain the amount of oxygen present at and multiplying again by $4\pi r^2$ we obtain the amount of oxygen present on th surface of a sphere with the radius now integrating with respect to r within the limits and we obtai the amount of oxygen present in the sphere this divided by πa^3 (volume of the sphere) and again by α gives the average pressure within the sphere, the result is

$$p_T(t) = \Delta P_{af} - \frac{3\,\Delta p_{af} D_t\ \beta}{\beta a^2}\left[1 - \left(\frac{\beta a^2}{D}\right)^{\frac{1}{2}} \cot\left(\frac{\beta a^2}{D}\right)^{\frac{1}{2}}\right] + \\ + \frac{6\,\Delta p_{af} \beta a^2}{\pi^4 D} \sum_{=1}^{\infty} \frac{-D_n \quad t/a^2}{n^2\left(n^2\pi - \frac{\beta a^2}{D}\right)} \tag{7 b}$$

It will be observed that the unknown has disappeared from (7 b) and the only unknowns are β nd D it is then enough to have a pair of two equations (7 b) and solving them w get β and D

Once β and D are known from (7 b only P remains to be calculated before p_t is determined from (5 P_{gt} as stated earlier $= p_{max}$ $\bar{p}_{T(t)}$) is experimentally determined to this value must be added the difference $p_{max} - \bar{p}_{T(t)}$) in order to give p_{max} itself. By definition $p_{max} = \bar{p}_{(t)} + \bar{p}_{T(t)}$) may also be derived from (2) and

$$\bar{p}_{T(t)}\ ) = \left[\frac{3}{4\pi a_3 \alpha} \int 4\pi r^2 \alpha (2a)\, dr\right] \quad = p_{(t)}\ ) - \frac{ma^2}{15\,D} \tag{16}$$

It follow that $p_{max} - \bar{p}_{T(t)}\ ) = \frac{ma^2}{15\,D}$ and therefore

$$P \ = \bar{p}_{T(t)}\ ) - \frac{ma^2}{15\,D} \tag{17}$$

For the rest of the calculations the procedure is the same as for the ideal, pointlike electrode.

The practical application

In order t get sufficient data to show how the theory outlined above may be pplied some preliminary measurements were done with the following methods.

The arterial and the subcutaneous po_2 were measured t the same time while a subject was given air or pure oxygen through mouthpiece.

The arterial po_2 was measured with the Clark electrode as described by ROOTH *et al* (1959) A plastic catheter was inserted into the brachial artery and blood samples were drawn t known intervals. The dead space in the syringes was filled with heparin. The blood was drawn as quickly as possible and the sample considered as taken when half the syringe was filled. Because of the oxygen consumption of the blood itself, the observed po_2 measurements were corrected for the time when the blood was drawn using the figures for the oxygen consumption of the whole blood given by DEWARDENER and YOUNG (1951)

The tissue po_2 was measured by inserting platinum electrode 0.2 mm in diameter bout 2 mm into the subcutis of the forearm as a cathode, while silver-silverchloride anode was placed on the skin a few cm. from the platinum cathode. 0 6 V was applied be tween the electrodes and the resulting current recorded by means of a LKB-Blomgren polarograph and Leeds & Northrup Speedomax recorder The platinum electrode was of the same type as that used by MONTGOMERY and HORWITZ but in addition it was covered with thin film of plastic, as suggested by BARTELS *et* *l*. (1959) The tip of the electrode was immersed in solution of 0.2 polystyrene in trichlorethylene and afterwards the electrode was dried for six hours at 60 C. This covering of the electrode made it much more stable and also made the results become more reproducible. If bleeding occurred during the insertion of the electrode this was replaced in another hole.

The calibration of the platinum electrode was made in Ringer solution of known po_2. The readings were not appreciably affected by stirring the solution and the same calibration was found valid for blood plasma. Recordings obtained in several healthy subjects breathing air gave values, calculated by this calibration, of about 40 mm Hg, which is the value arrived at by the use of the subcutaneous bubble technique (CAMPBELL 1924—25) W therefore used this calibration of the covered electrode in spite of the difference obtained in different media by MONTGOMERY (1957) and by ISCH (1958) using bare platinum electrodes However the calibration method is not ideal and it will be an advantage to the measurements once it is possible to obtain absolute readings.

In several experiments uncovered platinum electrodes were used and the time between the initiation of the oxygen breathing and the rise in the O_2 recording was the same as when the polystyrene covered electrode was used. The steepness of the curve was also similar Therefore, the covering of the electrode does not seem to affect the response time of the electrode.

The measurements could also be improved by taking due account to the time lag in the response of the *Pt* electrode and of the recorder

I order to be able to sol the equations, besides the data obtained from these po_2 measurements, figures are needed for *m* and *a*. For an estimation of the oxygen consumption this was measured on piece of subcutaneous tissue immediately after it was excised and was found to be 0 156 10^{-7}g sec g tissue This was measured polarographically with the dropping mercury electrod by analyzing the drop in po_2 caused by the metabolism of piece of tissue immersed in glucose Ringer solution at 37° C.

WETZEL and ZOTTERMAN (1926) give mean of 7 capillaries per mm in the subcutis of the forearm of man, which gives value of 0 007 cm for *a*.

Because of the inaccuracy of the calibration of the electrodes and of the figures *m* and and because no correction was made for the actual temperature t the point of po_2 measuremen in the tissue the value of the experiments described lies in the possibility of applying the equations for practical purpose and not in the physiological results obtained.

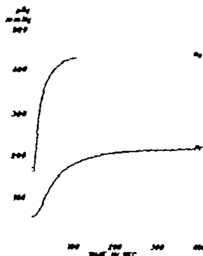


Fig 4 The increase in oxygen tension in the artery and in the subcutaneous tissue during oxygen inhalation. Case 1

Calculations

The changes in tissue and arterial oxygen pressure when a subject is switched over from breathing air to breathing pure oxygen is shown in Fig 4 The constants β and δ of equation (4) were calculated from the experimental findings by the method of trial and error

For the calculation of the oxygen diffusion coefficient only the tissue curve is needed. A set of two equations (7 b) is established with the experimental p_T values read off from the tissue curve together with the corresponding values for t. The values chosen for t must be such that p_T is > 150 mm/Hg in order to avoid any interference from an inconstant γ caused by unsaturation of the hemoglobin.

When writing down the constants, meticulous care must be taken to give all the data in the same unitary system. The C. G. S. system was here adopted for the calculations but the results are given in mm Hg

It was found con venient for the sake of the calculations to rewrite equation (7 b) by introducing $x = \beta a^2/D$ after which it assumes the form

$$A - \frac{B}{x} \quad A\left[1 - \sqrt{x}\cot\sqrt{x}\right] + \frac{2}{\pi^2}\sum_{1}^{\infty}\frac{e^{-n^2 \ldots}}{n^2(n^2\pi^2 - \ldots)} \quad 0 \qquad (7c)$$

where A and B are known figures.

Two limits were chosen for x so as to include all the physiologically possible values for D and within these limits the equations were solved. (D for the tissues should be lower than that of water which is $1.8 \quad 10^{-5}$) Also, $\beta < \delta$ because the tissue p_{O_2} cannot increase as rapidly as the arterial p_{O_2} which furnishes the increase to the tissue

In a diagram β two point by point lines are plotted, each one representing the solution of one of the tw (7) equations The points of intersection of the tw lines are the solutions of the tem. If several solutions are obtained each one has to be tried. It will then be found that only one single solution gives satisfactory figures such as positive values for the blood flow

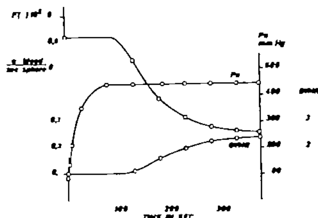


Fig. 5. The relation between arterial oxygen tension, the capillary blood flow and oxygen vasomotor reaction during oxygen inhalation. Case I

I order to reduce the time for solving set of equations (7) it was found convenient to tabulate the terms $\frac{1}{\ } \left[1 - \sqrt{\ } \cot \ \right]$ and $\frac{2}{\tau^4} \frac{1}{x - x}$ These tables may then be used for different sets of equations as the factors used are independent of the individual values of β and D

Equations (7) may then be solved by the use of common office calculator and long slide rule.

When inserting m in (10) and (17) it must be observed that in (10) m is expressed as g O consumed/sec./cc. tissue but in (17) it is expressed as decrease in oxygen tension/sec.

$\partial Q_1/\partial t$ is calculated from (10) and $\partial Q_2/\partial t$ is calculated from (11) By adding $\partial Q_1/\partial t$ and $\partial Q_2/\partial t$ we get $\partial Q/\partial t$

The capillary flow is calculated from (14) and in order to use this equation $p_{(a-c)}$ must be calculated. Now $p_{(a-c)}$ is given by (9) where all the figures are known except P which is obtained from (17)

Finally the vasomotor reaction is calculated according to (15)

As stated abov the flow is only calculated from the time when the tissue pressure is above 150 mm/Hg, when $p > 300 - 400$ mm/Hg and the hemoglobin is totally saturated and therefore γ = constant. The initial flow may be calculated by introducing γ valid for this time. This new γ^1 depends upon the oxygen dissociation curve. γ^1 actually consist of two parts the original γ and γ_m which is calculated as follows.

It must be determined how much oxygen the arterial blood releases when passing through the capillaries. The initial p is known and the initial p_c is already calculated from (5) The pH and the oxygen capacity must be measured and by the means of the oxygen dissociation curve the amount of oxygen released is calculated This amount, divided by the pressure drop between the artery and the capillary gives the average γ_m in that range of pressure. Finally $\gamma = \gamma + \gamma_m$ is introduced in (14)

Thus the initial flow is obtained. From this point to the point corresponding to $p_T =$ = 150 mm/Hg, the curv representing the flow was drawn by hand. The details of the curv from the beginning to $p_T = 150$ may if necessary be calculated with the procedure described.

Results

Taking oxygen curves from the subcutaneous tissue of the forearm of two healthy male subjects we found for case 1 values of $D = 0.90 \cdot 10^{-4}$ and case 2 $1.07 \cdot 10^{-}$ cm. /Sec.

These values are of the expected order of magnitude as compared with in vitro measurements in different tissues — THEWS and NIESEL (1958—59)

The arterial p_O the capillary flow and the vasomotor reaction are shown together in Fig 5 (case 1) Very similar results were obtained in case 2.

If the blood flow is recalculated in ml of blood per min. per 100 c. c. subcutaneous tissue, the figures are 17 for case 1 and 16 for case 2 This agrees with plethysmographic measurements such as those of FELDER *et al* (1954)

Discussion

The proposed mathematical treatment of the p_O curves from the subcutaneous tissues includes several assumptions, two not mentioned earlier are the constancy of the intercapillary distance (a) and the oxygen consumption of the tissue (m) It is possible that for exact measurements the figure for a may have to be determined in each case. If, for instance, m increases with increasing p_O the figures for the capillary blood flow would be higher and the figure for the vasomotor reaction would be lower This may have to be studied for each particular tissue and individual investigated. It follows that if a or m are variable, further mathematical treatment will be necessary but the preliminary figures obtained seem to indicate that the assumptions made in the present study are justified.

In the calculation of the oxygen diffusion coefficient the results obtained are more exact as m does not enter the equation.

The effect of oxygen inhalation itself is directly evident from Fig 5 It will be seen that about 2 min. after the inhalation of oxygen has started, the capillary flow is reduced and the gradual reduction continues for about 2 min. after the increase in p has stopped This is the effect of the vasomotor reaction working at the level of the arterioli. If the initial diameter of the arterioli is considered as 1 the final diameter is 0 80 in case 1 and 0 81 in case 2. In the brain the corresponding figures have been observed by THEWS (personal communication) to be 1 0.83

The method here described for studying the factors governing the oxygen flow to the tissues is specially suited for the subcutaneous tissue, because of the low oxygen consumption in relation to the blood flow but it may be used for other structures such as muscles.

The main value of the method for analyses of the p_T curves here presented lies in the possibility of measuring the oxygen diffusion coefficient in vivo.

Secondly the analyses have indicated which factors influence p_T curves obtainable with a platinum electrode.

The figures for the blood flow oxygen vasomotor reaction and praecapillary

constriction during oxygen breathing would be obtainable with other already established methods. But under experimental circumstances where one wants to measure the tissue oxygen tension, it must be an advantage to obtain values for blood flow and so on without the use of additional equipment except for analyses of the arterial oxygen tension.

At one stage in this work we tried to do the calculations by means of an electronic computer. These calculations were kindly performed at the Department of Mathematics by Prof. C. E. Fröberg. We also wish to thank those who have kindly read the manuscript and given important criticism, especially Dr. G. Thews, Kiel. This study has been supported by a grant from the Association for the Aid of Crippled Children, New York, U. S. A.

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From the Department of Physiology Karolinska Institutet, Stockholm, Sweden

Analysis of Prenatal Spinal Reflex Activity in Sheep

By

L. Ångoård R. Bergström and C. G Bernhard

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Abstract

L. Å ngoård, R. Bergström and C. G Bernhard *Analysis of prenatal spinal reflex activity in sheep* Acta physiol. scand. 1961 53. 128—136 — An electrophysiological analysis was made of the prenatal development of the reflex muscular response to stretch in 17 non-anaesthetized sheep fetuses (calculated gestational age 53 —132 days) kept in placental contact with the decerebrate ewe. Electrical muscl activity recorded with needle electrodes in the gastrocnemius muscle in response to stretch of the muscle ppeared around the 60th day and was shown to be of reflex order Antagonistic inhibition and inhibition evoked by skin stimulation were not found until later on, there being a short period in which no signs of central inhibition were obtained. Decerebrate rigidity was not obtained until the end of the gestation period, the total length of which is about 150 days in sheep

Introduction

In earlier investigations by e g Carmichael on the guinea pig (1934) and by Barcroft and Barron on the sheep (see review 1939) the prenatal de elopment of both spontaneous and reflex movements in response t different types of stimulation was studied by observing the movements. A closer study of the appearance and the development of different spinal reflex functions with electroph siological methods during the prenatal period has, however never been mad w th the exception of some preliminary observations made by Bernhard, K er and Kolmodin (1961) in connection with their study of induced cortical u ty in sheep fetuses during the last part of the gestational period.

Present address Dep ment of Physiolog University of Helsinki.

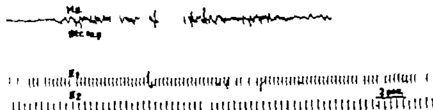


Fig. 1 Electromyogram from m. gastrocnemius (M. g) in response to stretch (Str m. g) in fetus with calculated age of 61 days. Lower tracings show the electrocardiograms of fetus (E_f) and ewe (E_e).

This investigation aims at the elucidation of the prenatal development of segmental reflex activity in the lumbosacral part of the cord. Special attention was paid to the relation between spinal excitation and inhibition and their interrelation in the functional development of reflex differentiation.

A preliminary report was given at the 10th Scandinavian Congress of Physiology in Oslo (ÅNGGÅRD, BERGSTRÖM and BERNHARD 1960)

Methods

The experiments were performed on sheep fetuses with weights from 12 g to 2,650 g, the ages of which (53—152 days) were calculated from the equation given by HUGGETT and WIDDAS (1951) The ewe was decerebrated under thiogenal, anaesthetized with d-tubocurarine and kept on artificial ventilation (cf BERNHARD, KAISER and KOLMODIN 1959) The fetus was delivered by caesarean section. Several fetuses with weights under 500 g were exteriorized in stages. Thus, the whole amniotic sac was first exposed, the spontaneous activity as well as certain reflexes and pulse rate of the fetus being observed under intra-amniotic conditions. In the next stage the fetus was exposed after rupturing the amniotic sac (see below) After exteriorization the fetus was mounted on a small table which was fixed close to the abdomen of the ewe and care was taken not to disturb the umbilical circulation. The fetus was kept in prone position on a bed of cotton wool and the preparation was covered with warm paraffin oil. One of the hind legs was fixed by means of needles through the cartilaginous tissue at the knee and ankle. The distal part of m. gastrocnemius was dissected free and by means of a thin thread attached to a lever by which the muscle could be passively stretched. In several preparations (see below) m. tibialis anterior was prepared in the same way The action potentials from muscles were recorded with needle electrodes. The muscle activity as well as the ECGs of the ewe and the fetus were recorded with a Grass electroencephalograph. The experiments usually started about hours after the injection of thiogenal, the experiments thus being performed on non-anaesthetized preparations. In several fetuses (see below) decerebration was performed at the end of the experiment in order to study the appearance of decerebrate rigidity during the prenatal period.

Dextran was given continuously to the ewe by intravenous drip and care was taken to keep the whole preparation at body temperature.



Fig 2 Electromyogram from *m gastrocnemius* (M. g.) in response to stretch (Str m. g) in fetus with calculated age of 60 days before spinal transection (I) after spinal transection at the upper lumbar level (II) and after spinal cord destruction (III) Sk. stim. marks skin stimulation of the foot ipsilateral to the muscle tested. Lower tracing (E_2) shows the ECG of the ewe.

Results

All fetuses used in this material showed spontaneous and reflex movements both when they were kept in the exposed unpunctured amniotic sac and after puncturing the sac. In the small fetuses the movements were, however reduced and less frequent after emptying the amniotic sac. The fact that these movements again became more pronounced after refilling th sac with Ringer solution indicates that the reduction of the movements after emptying the sac is not due to deterioration of the preparation but depending on unfavourable mechanical conditions. The muscle contractions of the small fetuses are obviously too weak to result in movements when the fetus is not swimming freely n the surrounding intra-amniotic fluid. The ECG recordings showed that in general the pulse rate of the small fetuses was about 20 per cent higher when the fetus as totally exteriorized than when it was kept in the unpunctured amniotic sac. All the experiments presented in this material were performed on fetuses whose heart rate was of the same magnitude as that obtained shortly after delivery

Fig 1 shows the electrical muscular activity of *m. gastrocnemius* (M g) in response to a slight stretch of the muscle (marked by the thick line in the third tracing) in a 35 g fetus (calculated age 61 days) As seen there is an irregular muscle activity during the whole period of stretch. This activity often is of recruiting character during the first period of stretch This is more clearly seen in Fig 2 I which shows the records from a fetus with a calculated age of 60 days. The activity was again recorded from the gastrocnemius muscle in response to stretch. In this experiment the spinal cord was then transected at the lower thoracic level and finally destroyed. The record in Fig 2 II taken after spinal transection still show a response (M. g) [illegible] tch although the activity is now much less pronounced destruct the lumbo-

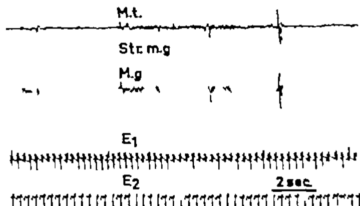


Fig 3. Electromyogram from *m. tibialis anterior* (M. t.) and *m. gastrocnemius* (M. g.) in response to stretch of the latter (Str m. g.) in fetus with calculated age of 67 days. Lower tracings show the ECGs of the fetus (E_1) and the ewe (E_2).

sacral spinal cord the response to stretch disappeared totally as seen in Fig 2 III Experiments of this type show that the muscular response to stretch is dependent on an intact spinal cord. As seen in Table I the muscular activity in *m. gastrocnemius* in response to stretch was tested in 17 fetuses of varying ages and was obtained in all fetuses older than 58 days, whereas no such response could be obtained in the younger fetuses tested (ages 53—58 days)

In 14 fetuses out of 17 preparations tested, the activity in *m. tibialis anterior* was recorded simultaneously The upper and third tracings in Fig 3 show the activity picked up by a needle electrode in *m. tibialis anterior* (M. t.) and *m. gastrocnemius* (M. g.) respectively in a fetus having a calculated age of 67 days. As seen a stretch of the gastrocnemius muscle (Str m. g.) is followed by activity not only in the gastrocnemius muscle but also in *m. tibialis anterior* This type of response to stretch of *m. gastrocnemius* was obtained in all fetuses tested with ages between 60 and 81 days. In some of these fetuses stretching of the gastrocnemius muscle was performed also in periods when there was a background activity in *m. tibialis anterior* and vice versa. In these experiments signs of antagonistic inhibition were never seen but always signs of excitation of the antagonist, as illustrated in Fig 3. The youngest fetus in which signs of antagonistic inhibition were observed had a calculated age of 90 days. An experiment on this fetus is illustrated in Fig 4 The first record shows how the activity in *m. gastrocnemius* evoked by stretch (Str m. g.) diminishes when a slight stretch is applied to *m. tibialis anterior* (Str t.) The activity in *m. tibialis anterior* in response to stretch is shown in the third tracing (M. t.)

In most fetuses (15 out of 17) the effect of ipsilateral skin stimulation (pinching the skin of the foot) on the activity in *m. gastrocnemius* was investigated.

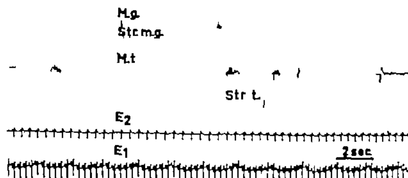


Fig. 4. Electromyogram from *m. gastrocnemius* (M. g.) and *m. tibialis anterior* (M. t.) in response to stretch of these muscles. (Str. m. g. and Str. t.) in fetus with a calculated age of 90 days. E_1 and E_2 show the ECG:s of the fetus and the ewe respectively

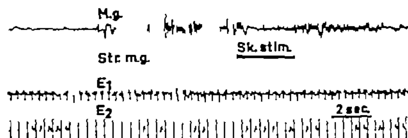


Fig. 5. Inhibition by ipsilateral skin stimulation (Sk. stim.) of the activity in *m. gastrocnemius* (M. g.) evoked by stretch of the muscle (Str. m. g.) in fetus with calculated age of 72 days. E_1 and E_2 show the ECG:s of the fetus and the ewe respectively

In the experiment illustrated in Fig. 2 I made on a 60-day fetus, ipsilateral skin stimulation (Sk. stim.) had no inhibitory effect on the activity in *m. gastrocnemius*. In the fetuses of this age skin stimulation was instead followed by an increase of activity. The earliest age at which ipsilateral skin stimulation was found to inhibit the activity in *m. gastrocnemius* was 72 days. This effect is illustrated in Fig. 5. The upper tracing shows the slowly increasing activity in *m. gastrocnemius* in response to stretch (Str. m. g.) which was inhibited by pinching the skin between the cloves on the same side (Sk. stim.). As seen in Table I, inhibition in response to ipsilateral skin stimulation was obtained in 7 fetuses out of 9 tested with ages above 69 days. Table I thus shows that whereas reflex excitation of the motoneurones evoked by stretching the muscle could be obtained around the 60th day, signs of classical inhibition (due to stretch of the antagonistic muscle or to ipsilateral skin stimulation) could not be obtained until later on during the prenatal period. Table I also shows that signs of reflex inhibition evoked either from antagonist or by skin stimulation could be demonstrated in 8 out of 10 fetuses tested with ages above 69 days.

Table I

Weight, g	Calculated age, days	Decerebrate rigidity	M. gast. response to stretch	Inhib. of M. gast. response by ipsilat. skin stim.	Inhib. of M. gast. response by stretch of M. tib. ant.
12	53	−	−	−	−
20	57	−	−	−	−
25	58		−	−	−
30	60	−	+	−	
35	61		+	−	−
90	69	−	+	−	−
126	72		+	+	−
250	81	−	+	+	−
440	90		+	−	+
500	92	−	+	+	−
885	103		+	−	−
1,570	117	−	+	+	−
1,670	119	−	+		
1 750	120	(+)	+	+	
1,850	122	−	+	+	−
2,300	127	−	+	+	+
2,650	132	+	+		−
	Newborn	+	+	+	+
	Adult	+	+	+	+

The effect of decerebration at different prenatal stages was also investigated In this material 12 fetuses with varying calculated ages from 53 to 132 days were decerebrated. As mentioned above, decerebration was performed after the reflexes described above had been tested. Clear signs of decerebrate rigidity were only found at the end of the prenatal period (see Table I) In newborn lambs decerebrate rigidity was regularly obtained as in the adult sheep. In

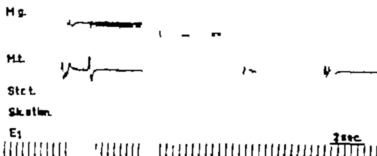


Fig. 6. Effect of ipsilateral skin stimulation (Sk. stim.) and stretch of m. *tibialis anterior* (Str t.) on the electromyograms from m. *gastrocnemius* (M. g.) and m. *tibialis anterior* (M. t.) in decerebrate preparation of newborn lamb. E_1 shows the ECG.

some of the younger fetuses the different types of reflex activity described above were tested and found to be unchanged after decerebration.

Fig. 6 shows electromyograms from *m. gastrocnemius* and *m. tibialis anterior* in a decerebrate newborn lamb. The typical tonic discharge in the extensor muscle (M. g.) characteristic of the decerebrate state was never obtained in the fetuses before the end of the gestation period. Fig. 6 also shows the inhibition of the tonic discharge following a slight stretch of *m. tibialis anterior* (Str. t.) the reflex response of which is shown in the third tracing.

Discussion

It was found that muscle activity in response to stretch could not be evoked until about the 60th gestational day in the sheep, the total fetal period of which is about 150 days. It has been claimed that stretching a fetal muscle may act as a stimulus for the contraction of the fibres in the absence of the spinal cord (MINKOWSKI 1928). The fact that the muscular response described above was found to disappear after destruction of the lumbosacral part of the cord shows that this response to stretch is to be regarded as a spinal reflex. The question then arises if this "fetal myotatic reflex" which appears around the 60th day is evoked by stimulation of proprioceptors. In this context it should be pointed out that according to histological investigations (DICKSON 1940) simple definite spindles appear around this time in sheep. A comparison of our electrophysiological observations with these histological data would indicate that the "fetal myotatic reflex" demonstrated can be evoked from muscle spindles and that the lack of this type of reflex before the 60th day may be due to an incomplete development of this receptor function. So far we have not made latency measurements of the nerve or motor root reflex responses to electrical stimulation of afferent nerves at this early stage in order to obtain evidence for the existence of monosynaptic reflex transmission. In connection with other investigations (BERNHARD, KAISER and KOLMODIN 1959) such experiments were made on older fetuses which indicated the existence of a prenatal monosynaptic reflex transmission in sheep fetuses during the last part of the gestation period. In this context it should be mentioned that in newborn kittens proprioceptive reflexes are mediated by two-neuron arcs (SKOGLUND 1960 b).

At the fetal age of 60 days stretch is obviously followed by excitation not only of the motoneurones innervating the same muscle but also other muscles and even antagonists. Further, the experiments indicate that at this stage reflex excitation dominates since no signs of reflex inhibition following proprioceptive and exteroceptive stimulation could be found until the 72nd day. Thus, there seems to be a fetal period during which there is a pronounced lack of balance between the excitatory and inhibitory mechanisms which may explain the diffuse and widespread character of the reflex activity. The fact that during this period no signs of inhibition could be obtained as the result

of exteroceptive and proprioceptive stimulation, although both types of stimulation evoke widespread reflex movements, indicates that the absence of inhibition is of central order This conclusion is in accordance with the results of spinal reflex experiments on newborn cats (MALCOLM 1953 and 1955) the functional central nervous maturation of which in relation to birth is delayed compared with the sheep. MALCOLM did not find any signs of spinal inhibition in the newborn cat. On the other hand, SKOGLUND who also investigated the postnatal development of spinal reflex activity in kittens found inhibition of extensors from flexors to be potent and long-lasting (1960 d) and referred the different results in newborn kittens to variations in maturity of the kittens at birth (1960 a, b and c)

It should be mentioned that muscular activity can be evoked by exteroceptive stimulation at earlier fetal ages (in sheep from about the 35th—40th day) which activity seems to be of reflex order the trigeminal region being the most sensitive reflexogenic zone in the earliest stages (see BARCROFT and BARRON 1939) The response to stimulation of the skin, e g on the nose or on a leg in these young fetuses develops into a generalized reflex movement which according to BARCROFT and BARRON eventually becomes restricted to the muscles of the region stimulated. We have also studied this early generalized type of fetal reflex response to exteroceptive stimulation under intra-amniotic and extra-amniotic conditions. In general it was found that the generalized response pattern faded at the stage when the balance between excitation and inhibition appeared in the segmental reflex tested. According to the data given by BARCROFT and BARRON (1939) who only observed the movements following different types of stimulation, diffuse reflex responses to proprioceptive stimulation should appear somewhat earlier (48th day) than found in our electrophysiological investigations (60th day) but since they used flipping of the limb as stimulus, exteroceptive stimulation may have been involved in their experiments.

Finally both the fetal myotatic response and peripherally evoked inhibition of this response were observed long before the appearance of decerebrate rigidity characterized by the sustained tonic muscle activity which was obtained in the last part of the prenatal period and in the newborn lamb. It is interesting to note that in newborn kittens no tonic reflexes can be obtained from the muscles concerned with decerebrate rigidity (SKOGLUND 1960 a) In this animal, the discharge of receptors in the gastrocnemius muscle in response to stretch was found to be phasic at birth, according to recent investigations by SKOGLUND (1960) who also concludes that post tetanic potentiation plays an essential role for the appearance of the tonic stretch reflex (1960 e)

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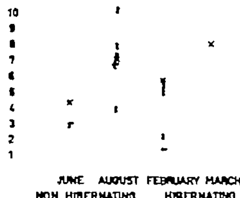


Fig 1 shows the myocardial lactate concentration in hedgehogs

aspect the authors have determined the lactate concentration in myocardial tissue in a non-hibernator the guinea-pig at normal body temperature and during hypothermia and in a hibernator the hedgehog, during the summer when the animals were not hibernating and during hibernation in the winter

Material and methods

For the experiments w used 45 hedgehogs (Fig 1) — 23 in a non-hibernating state of which 6 in June and 17 in August, and 22 in a hibernating stat of which 19 in February and 3 in March — and 49 guinea pigs (Fig 2) Of the guinea-pigs 10 were used as controls 15 were cooled to 30 C, 4 of which were kept t 30 C for tw hours 7 animals were also cooled to 30 C but in contrast to the preceding group of 15 guinea-pigs these animals received artificial respiration from temperature of 32—34 C 5 animals ere ooled to 25 C and kept t this temperature for two hours receiving artificial respiration during th whole experiment 11 animals were cooled to 20 C receiving rtificial respiration from temperature of 32 —34 C 5 nimals were cooled while receiving artificial respiration and then at 30 C the aorta and both venae cavae were clamped for 8 min The hedgehogs were decapitated when hibernating while the non-hibernating animals were first anesthetized w th nembutal. During cooling the guinea-pigs were also sufficiently anesthetized w h nembutal as to inhibit shivering The artificial respiration as performed with pure ox gen through cannula in the trachea

An enzymatic method described by Hohorst (1957) and slightl modified bu w as used for th lactat determinations At the end of each experiment the heart was immediately removed, rinsed in old water for a few seconds then quickly frozen by placing it in a small glass tube which was surrounded with solid carbon dioxide

The lactat concentration was determined on wet tissue The lues were expressed as mg/g dry weight T obtai he elation between dry nd wet weights specimens of about 0.5 g tissue were d d temperature of 105 C for 2 hours nd then kept in a vacuum desiccator with phosphorous pentoxid over night.

The t analysis test was used for calculating possibl d fferences between the series A p value of 0 01 or less is regarded as significant

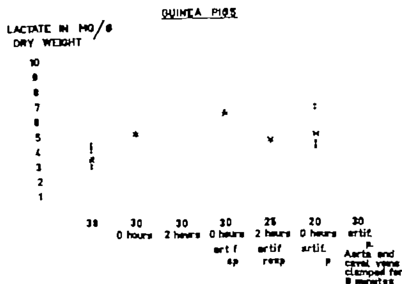


Fig 2 shows the myocardial lactate concentration in guinea-pigs.

Results

The results are given in Fig 1 and 2. The range of the values obtained in the *hedgehogs* was rather wide especially in animals hibernating in February with values varying from 1 4 to 10 4 mg/g dry weight. No significant differences were found between the separate series.

There was a lower lactate concentration in the myocardial tissue from the control *guinea-pigs* in which the samples were taken just after anesthesia at normal body temperature than in the hypothermic animals ($0.01 < p < 0.02$) Animals kept for 2 hours at a body temperature of 30° C showed no significant difference from samples taken from animals that were killed immediately after they had reached 30° C. Nor was there any distinct difference ($p = 0.02$) between animals that had received artificial respiration at 30° C and those who had not. When the temperature was decreased further the lactate values did not change but remained on about the same level as at 30 C. The value obtained in animals in which the circulation had been clamped for 8 min was significantly higher than the control value ($0.001 < p < 0.01$) and also higher than the value obtained at 20 C ($p = 0.01$)

Discussion

This study was made to try to establish whether there is a difference in the metabolism between hibernators during the hibernating and non-hibernating period — *some facts speak in favor of this* — *and to see how a non-hibernator* reacts metabolically to a lowered body temperature. It is obvious from Fig 1

ALDOLASE UNITS

32

28

24

20

16

12

8

4

0

FEBRUARY 1958 AUGUST 1958

Fig. 3. shows the serum aldolase concentration in hedgehogs in a hibernating state in February and in non-hibernating state in August.

that as far as lactate is concerned there were no great changes in the hibernators. We also studied the serum aldolase concentration in hibernating (February) and non-hibernating (August) hedgehogs. As shown in Fig. 3 the mean value during hibernation was higher than that obtained in the summer. There are, however, rather few values and two in the February series are unusually high. If these two values are omitted and if one takes into consideration the higher hematocrit values in the hibernating animals (BIÖRCK, JOHANSSON and VEIGE 1956) it is doubtful whether there is any profound difference in serum aldolase concentration in hibernating and non-hibernating hedgehogs.

The lactate content is considered a reliable indicator of the degree of anaerobic metabolism in a tissue. As it has been claimed that hypothermia produces an anoxic state in the myocardium we thought it of interest to see if this possible anoxia was revealed in an increased lactate concentration in the tissues. Although the control values were lower than the values obtained at hypothermic levels this difference showed only a borderline significance. Even if the animals apparently breathed well at 30°C we administered artificial respiration to some of them (see Fig. 1). This, however, did not decrease the lactate level. When the animals were cooled to 20° C and 25° C and kept at these temperatures for 2 hours the lactate content did not increase. There was rather a contrary tendency when comparing with the values obtained at 30° C.

The concentration in blood of lactate during hypothermia has been reported by different authors. WADDELL, FAIRLY and BIGELOW (1957) obtained some

Aldolase was chosen because it is one of the more important enzymes involved in the anaerobic metabolism of carbohydrates. Aldolase was determined according to method by Sibley and Lehninger (1949)

elevation of blood lactate after the induction of anesthesia and hypothermia in man but no major changes occurred during cooling. In dogs KUHN *et al.* (1959) observed no change in the lactate concentration during cooling to 17° C. Both these teams found a marked increase of the lactate level after surgery and circulatory occlusion and following rewarming. THORN *et al.* (1958) found no change in lactate concentration in brains from rabbits at 37° C and 26° C, nor were any changes observed in other organs examined including the heart. However, if the brains were rendered anoxic or ischemic there occurred a marked increase in the lactate content.

Our results are in conformance with those published by THORN *et al.* (1958). The increase we noted at 30° C might be explained by a subclinical shivering lactate proceeding into the myocardium as a result of the increased serum lactate concentration. It is not caused by anoxia due to insufficient respiration since artificial respiration with pure oxygen did not change the result. The hypothermia per se is probably not the cause of the increase (which shows only a borderline significance) for there is no further increase with lowering of the temperature.

The electrocardiographic changes observed during hypothermia have been interpreted as being of an anoxic nature (LANGE *et al.* 1949). The present investigation does not support this view since the increase in lactate concentration is only of marginal significance and does not increase with lowered temperature as do the electrocardiographic changes.

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We gratefully acknowledge the technical assistance by Miss ANNA-LENA SAMUELSSON.

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From the Departments of Neurology and Pediatrics of the Harvard Medical School and the Neurology and Children's Medical Services and the Joseph P. Kennedy Jr. Memorial Laboratories of the Massachusetts General Hospital, and the Anesthesia Laboratory of the Harvard Medical School at the Massachusetts General Hospital, Boston, U.S.A.

Studies in Neuromuscular Transmission

I. Influence on Neuromuscular Transmission of Alkalosis and Acidosis

By

INGRID GAMSTORP[1] and ERIK VINNARS[2]

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Abstract

GAMSTORP, I. and E. VINNARS. *Influence on neuromuscular transmission of alkalosis and acidosis.* Acta physiol. scand. 1961. 53. 142—150. — The effect of alkalosis and acidosis on the neuromuscular transmission was studied in rabbits. The sciatic nerve was stimulated through bipolar electrode pairs and the electrical response of the gastrocnemius muscle was recorded. The stimulus necessary to evoke a maximal response, neuromuscular conduction time, nerve conduction time, amplitude and duration of response, refractory and unresponsive periods were measured as was the response to repeated stimulation at a rate of 3 per second. Respiratory alkalosis was produced by hyperventilation, respiratory acidosis by administration of 5, 10 or 20 % carbon dioxide in oxygen, metabolic alkalosis by i. v. injection of 0.5 N sodium carbonate and metabolic acidosis by i. v. injection of 0.5 N hydrochloric acid. A slight decrease in amplitude of the response was the only change noted during metabolic alkalosis. No significant alteration occurred during respiratory alkalosis or metabolic acidosis.

[1] Jerry Lewis Fellow of the Muscular Dystrophy Associations of America, Inc. Clinical and Research Fellow in Neurology and Pediatrics, Massachusetts General Hospital and Harvard Medical School. Present address: Dept. of Pediatrics, Lund, Sweden.

[2] Visiting Lecturer in Anesthesia, Massachusetts General Hospital and Harvard Medical School.

Tw changes were observed, occasionally after 5 and 10 % carbon dioxide, almost regularly after 20 carbon dioxide, i. at pH below 7 and pCO abov 120 mm Hg I An increase in the stimulus necessary to evoke maximal response. II. A slight prolongation f neuromuscular conduction time. These changes were not caused by release of adrenaline.

Changes in carbon dioxide tension and blood pH re known to influence the action of neuromuscular blocking agents in clinical anesthesia (DUNDEE 1952, SCURR 19.4 GRAY and FENTON 1954) PAYNE (1958, 1960) and JOHANSEN and OSGOOD (1960) examined this influence experimentally in cats by recording the mechanical muscle response to nerve stimulation W th the same technique PAYNE (1958, 1960) also studied the effect of changes in carbon dioxide tension and blood pH on neuromuscular transmission in cats which received no neuromuscular blocking agents with variable results. Th following experiments were designed to obtain further information on the effect of alkalosis and acidosis on neuromuscular transmission by employing a more precise technique of measurement.

Material and Methods

Thirty-five albino rabbits weighing 2.0 to 4 1 kg were used. Fourteen (group A) were exposed to respiratory alkalosis and acidosis, 7 (group B) used as a control study of group A, were given epinephrine i. and 14 (group C) were exposed to metabolic alkalosis and acidosis. Pentobarbital sodium (28 mg per kg) was given i. v for the operative procedure about 2 hours before the experiment started. Procaine chloride locally was the only other anesthetic administered. The right femoral artery and vein were catheterized. A tracheal cannula was inserted through a tracheotomy in the rabbits belonging to group A to facilitate hyperventilation and the administration of carbon dioxide. The left sciatic nerve was exposed along its entire length and crushed and ligated as far proximal as possible. Bipolar stainless steel electrodes were used with the cathode placed most distally the distance between anode and cathod being 5 mm. Tw equal pairs of electrodes were applied to the nerve 3 to 6 cm apart, one close to the muscle and the other as far proximal as possible. The electrodes were sutured in place to prevent sliding along the nerve. The divided gluteal muscles were sutured and the skin closed. The rabbit was strapped to table of appropriate size and the left leg was secured with adhesive tape.

The stimulus was square wave pulse of 10 to 50 μsec duration. The oltage was increased un il maximal response was obtained and then increased about 25 abov this value (15—150 V). At this point it was always ascertained that a prolongation of the stimulus did not raise the amplitude of the response. The stimulus was maintained supramaximal and the necessary duration and oltage were recorded throughout the experiment. When double independent impulses with variable interval were used, the second stimulus was about 25 stronger than the first.

The electrical response in the left gastrocnemius muscle was recorded through concentric needle electrode on Disa electromyograph. The permanent recording of interrupted sweeps was made on photographic paper with sweep speed of 2 m per sec. The stimulator triggered the sweep of the electromyograph thus making it possible t get the stimulus artefact and the response on the same record. At the start of the experiment, the needle electrode was adjusted until simple response was obtained with

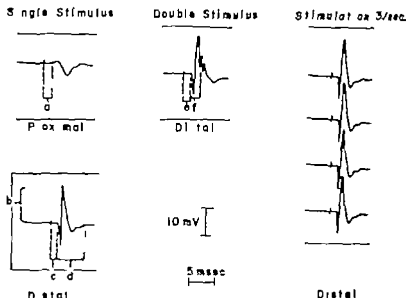


Fig. 1 Rabbit no. 37 Electrical response from l gastrocnemius muscle after supramaximal stimulation of sciatic nerve. Initial recording. = neuromuscular conduction time, a—c = nerve conduction time, b = amplitude of response, d = duration of response. = absolute refractory period f = absolute irresponsive period.

the highest possible amplitud and a thin, sharp initial deflection. The electrode was then fixed in this position with collodium and adhesive tape.

The following parameters were measured (Fig 1)

1 The voltage and duration of the stimulus needed to give a maximal response As a slight increase of the stimulus was occasionally necessary during prolonged experiments, the needed change in voltage had to be at least 20 V with unaltered duration, to be considered significant.

2 Neuromuscular conduction time the time interval from the beginning of the stimulus applied through th distal electrode pair to the beginning of the response (Fig 1) Smaller changes than 0.2 msec could not be measured accurately

3. Nerve conduction time th time interval from the beginning of the stimulus applied through the proximal electrode pair to the beginning of the response minus the neuromuscular conduction time (Fig 1 a—c) Smaller changes than 0.2 msec could not be measured accurately

4 The amplitude and duration of the response (Fig 1 b and d) For no apparent reason the amplitud of the potential could change 0.5—1 mV without an alteration of the shape of th response. If n obvious variation of the shape of the response occurred, indicating a shift in the position of the electrode, the experiment was discarded. Changes in the duration smaller than 0.5 msec could not be measured accurately

5. The absolute refractory period the shortest time interval between two supramaximal stimuli allowing both to evoke a response (Fig 1) Smaller changes than 0.2 msec could not be measured accurately

6. The absolute irresponsive period the shortest time interval between the responses evoked by two supramaximal stimuli (F g. 1 f) Smaller changes than 0.5 msec could not be measured accurately

7 The response to repeated stimulation at a rate of 3 per sec. The amplitude of the response did not change during repeated stimulation unless a competitive neuromuscular block was produced.

In order to maintain normal blood volume dextran was infused i. v. in amounts roughly corresponding to the blood loss. Arterial blood pressure was measured constantly with an aneroid manometer connected to the catheter in the femoral artery and maintained at a level above 60 mm Hg. The intramuscular temperature was measured by a telethermometer. It varied between 41 and 45 C, not changing more than 1 .5 during single experiment.

Respiratory alkalosis was produced by mechanical hyperventilation of the rabbit. The respirator used was positive-negative pressure pump with which tidal volume was increased 2.5 to 3 times the level known to maintain normal blood pH and carbon dioxide tension. Hyperventilation was continued for 10 min. Respiratory acidosis was produced by administration of 5, 10 and 20 % carbon dioxide in oxygen, each concentration for period of 10 min. Normal entilation with air for at least 10 min preceded the initial recordings. Arterial blood was drawn after each 10 min period.

Administration of carbon dioxide is known to cause release of epinephrine (Sechzer et al. 1960) which might influence the neuromuscular transmission (Goffart 1952, Bowman and Zaimis 1958). As the blood pressure usually showed slight increase (from 90—100 to max. 120—130 mm Hg) during administration of 20 % carbon dioxide, the following experiments were performed on the rabbits in group B. Epinephrine (5 μg/ml) was injected i. v. for 10 min with speed of 10 to 12 μg/min (= 3 to 5 μg/min/kg) which was considered to be above the amount released during severe stress (Bowman and Zaimis 1958). Recordings were made before during and up to one hour after the injection. A few samples of arterial blood were drawn.

Metabolic alkalosis was produced by i. v. injection of 0.5 N sodium carbonate solution; 8 to 9 meq were given over period of 2 to 3 min. Metabolic acidosis was produced by i. infusion of 0.5 N hydrochloric acid 5 to 10 meq were given over period of 2 to 4 min. In 2 experiments a second injection was given after completing the first one maximum of 13.5 meq was given. The total amount and the speed of the injection were adjusted so that significant drop in blood pressure was avoided. Arterial blood was drawn before the injection, one minute after the end of the injection and 5 to 15 min later

The blood samples were analyzed for carbon dioxide content by the method of van Slyke and the pH of the arterial blood was determined using Beckman pH-meter The partial pressure of carbon dioxide in arterial blood was calculated from the Singer Hastings nomogram. The concentration of potassium in plasma was measured using a Beckman flamephotometer T exclude the possible influence of the blood loss, 3 experiments with respiratory alkalosis, 5 with respiratory acidosis, 2 with infusion of epinephrine and 1 with metabolic alkalosis were performed without sampling of blood.

Results

Group A. Two severely anoxic rabbits were excluded from consideration. Results of analyses of blood samples from 5 of the animals with alkalosis and from 6 of the animals with acidosis showed that pure or almost pure respiratory alkalosis and acidosis were produced with no significant change in plasma potassium. During respiratory alkalosis the pH in the arterial blood was between 7.61 and 7.79 with the partial pressure of carbon dioxide between < 10 and 18 mm Hg. Corresponding values during respiratory acidosis were

after 5 % carbon dioxide 7.26—7.30 and 48—54 mm Hg after 10 % carbon dioxide 7.02—7.20 and 62—92 mm Hg and after 20 % carbon dioxide 6.83—6.98 with the carbon dioxide tension invariably above 120 mm Hg. All plasma potassium values were between 2.2 and 4.6 meq/l and the maximal change during one and the same experiment did not exceed 1.5 meq/l.

The results of the neuromuscular measurements are summarized under the headings defined in the section of methods.

Respiratory alkalosis.

1—7. No significant influence on the neuromuscular transmission was found in the 8 exp.

Respiratory acidosis.

1. The stimulus necessary to evoke a maximal response was found to be increased in 7 of 8 exp. after 20 % carbon dioxide, in 4 of 7 after 10 % carbon dioxide and in 4 of 7 after 5 % carbon dioxide. In 5 of 5 exp. the stimulus necessary to evoke a maximal response again decreased after normal ventilation with air. One rabbit was exposed to 20 % carbon dioxide for a second period of 10 min which again raised the stimulus necessary to evoke a maximal response.

2. The neuromuscular conduction time increased 0.2 to 0.6 msec in 9 of 11 exp. after 20 % carbon dioxide and did not change significantly in the other 2. In 4 of 4 exp. it returned to its initial value on normal ventilation with air. One rabbit was exposed to 20 % carbon dioxide for a second period of 10 min which again produced a 0.3 msec increase in neuromuscular conduction time. After 10 % carbon dioxide neuromuscular conduction time increased by as much as 0.2 msec in only 1 of 11 exp. and after 5 % carbon dioxide in only 1 of 9 exp. It did not change significantly in the other exp. (Fig. 1 and 2).

3. Nerve conduction time decreased 0.3 msec in one experiment and did not change significantly in the other 6. However, this figure being the small difference between 2 larger numbers is a less accurate measurement than the neuromuscular conduction time.

4. Provided the stimulus was maintained supramaximal by increasing it when necessary, a significant decrease in the amplitude of the response was recorded in 2 exp. only. An increase was never found. The duration of the response did not change.

5. No constant change in the absolute refractory period was found.

6. No constant change in the irresponsive period was found.

7. Subsequent responses to repeated stimulation at a rate of 3 per second showed no decrease in amplitude as compared with the first one.

The results of the experiments performed without sampling of blood did not differ from those in which blood was obtained.

Group B. Analysis of blood samples drawn before and immediately after injection of epinephrine in 3 exp. showed that no significant changes had occurred. The results of the neuromuscular measurements are summarized under the headings defined in the section of methods.

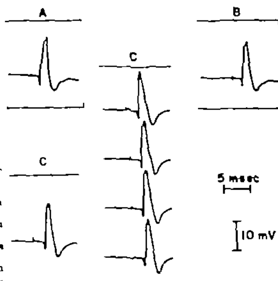


Fig. 2. Rabbit no. 37 same experiment as in Fig. 1; only distal stimulation.
A. Recording after administration of 5 % carbon dioxide for 10 min.
B. Recording after administration of 10 % carbon dioxide for 10 min.
C. Recording after administration of 20 % carbon dioxide for 10 min.
Lines on the illustrations have been darkened for photographic purposes.

1 The stimulus necessary to evoke a maximal response was found to be increased during the injection of epinephrin in 2 of 7 exp

2—7 No significant influence on the neuromuscular transmission was found in the 7 exp.

Group C During metabolic alkalosis the pH of the arterial blood increased to between 7.53 and 7 70 and the partial pressure of carbon dioxide was between 28.5 and 35 mm Hg (3 exp) The plasma potassium remained essentially unchanged. During metabolic acidosis the pH decreased to between 6 82 and 7.22. In 5 exp. it was below 7 in 2 7.00 and in 3 above 7 The partial pressure of carbon dioxide was between 21 and 39 mm Hg *In all of the experiments a* slight increase (0.5—3 meq/l) of plasma potassium was found, the highest noted value being 6.5 meq/l, which is below the level necessary to induce changes in neuromuscular transmission (Gamstorp and Vidkare, 1961)

The results of the neuromuscular measurements are summarized under the headings defined in the section of methods.

During metabolic alkalosis.

1 No significant change was noted in the stimulus necessary to evoke a maximal response.

2. Neuromuscular conduction time decreased 0.2 msec in 1 exp and did not change in the other 3

3 No change in nerve conduction time was found.

4 The amplitude of the response decreased 1 to 3 mV in all 4 exp. The duration of the response did not change.

5—7 No changes were found

During metabolic acidosis.

1 The stimulus necessary to evoke a maximal response increased in 2 of 10 exp

2. Neuromuscular conduction time increased with as much as 0.2 msec in only 1 of 10 exp.

3 Nerve conduction decreased 0 4 msec in 1 exp and did not change in the other 9

4 The amplitude of the response decreased 1 to 6 mV in 4 of 10 exp. The duration of the response did not change.

5 Absolute refractory period increased 0.3 msec in 1 of 10 exp. and did not change significantly in the other 9

6 Absolute irresponsive period increased 1 msec in 1 of 10 exp. and did not change significantly in the other 9

7 Subsequent responses to repeated stimulation at a rate of 3 per second showed no decrease in amplitude as compared with the first one.

Discussion

With the methods used respiratory alkalosis produced no measurable change in neuromuscular transmission. The only constant finding in metabolic alkalosis was a decrease in the amplitude of the response. Although slight, this change was noted in all the experiments. We have no explanation for this observation.

Neuromuscular transmission was altered in 2 ways during carbon dioxide inhalation An increased stimulus was needed to evoke a maximal response and neuromuscular conduction time was prolonged The first alteration occurred in all but one of the experiments in which the pH of the arterial blood was below 7 and carbon dioxide tension above 120 mm Hg and in half of those in which the acidosis was less severe and was found to be reversible. If the stimulus was maintained supramaximal no constant change in amplitude of the response was found. In his experiments on cats, PAYNE (1958) found the height of the muscle twitch to decrease during carbon dioxide inhalation. There are several possible explanations for this discrepancy First, different species were used and secondly PAYNE recorded the mechanical and we the electrical response to nerve stimulation PAYNE does not give the number of experiments upon which his conclusions are based. Finally although the strength and duration of the stimulus is given he does not state whether it was maintained supramaximal. Thus PAYNE's results might be explained by the same increase in threshold to maximal stimulation as was found in our experiments. It is of interest that as early as 1895 WALLER described a change in the excitability of the nerve during exposure to carbon dioxide. On isolated mammalian nerve exposed to different concentrations of carbon dioxide, LEHMANN (1937) found nerve excitability to decrease during acidosis.

The results of the experiments with epinephrine show that although the release of epinephrine may play a role it is not the whole explanation for the increase n the stimulus needed to evoke a maximal response.

The second of the alterations in neuromuscular transmission occurring during administration of carbon dioxide, namely prolongation of the neuromuscular conduction time was found in 9 of 11 exp. in which the pH of the arterial blood was below 7 and the carbon dioxide tension above 120 mm Hg but in only one of those in which the acidosis was less severe This was probably not due to a reduction of conduction velocity since conduction time in the nerve itself was found to be unchanged. Conduction time, like duration of th response, refractory and irresponsive periods, cannot be analyzed unless the electrical response is recorded. To our knowledge there are no data in the literature with which to compare our results. A prolongation of the neuromuscular conduction time is also found in hyperpotassemia (GAMSTORP and VIDOLARS 1961) However this cannot be the responsible mechanism in our ex periments, since little or no increase in plasma potassium occurred. Moreover the other characteristic findings of hyperpotassemia prolonged duration of the response and increased refractory and irresponsive periods (GAMSTORP and VIDOLARS 1961) were not found during administration of carbon dioxide. A release of epinephrine cannot explain the change in neuromuscular conduction time. The normal response to epeated stimulation excludes a block of the type produced by curare.

Administration of carbon dioxide characteristically causes a decrease in pH and an increase in carbon dioxide tension. In order to distinguish between those 2 factors, the series of experiments were carried out in which metabolic acidosis was produced. As changes in neuromuscular transmission were noted mainly during severe respiratory acidosis, our aim was to produce a metabolic acidosis with the pH of the arterial blood below 7 This was successfully done in 5 exp. and 2 additional ones the pH decreased to 7 In no instance did the partial pressure of carbon dioxide in arterial blood increase significantly

Measurable changes in neuromuscular transmission during metabolic acidosis were few and inconstant. In none of the 7 exp. with pH at or below 7 did the stimulus necessary to evoke a maximal response increase, in only one was a slight increase in neuromuscular conduction time noted, in 2 a drop in amplitude, in none a prolongation of refractory period and in on prolongation of irresponsive period. On the other hand in the 3 exp. with less severe acidosis, 2 showed n increase of the stimulus necessary to evoke a maximal response, 2 a prolongation of refractory period. These findings could not be related to changes in plasma potassium.

In hyperpotassemia, with or without secondary acidosis, the findings were a prolongation in neuromuscular conduction time, increases in duration of response, refractory and irresponsive periods and a decrease in amplitude these changes were not only of regular occurrence but larger than those noted

in metabolic acidosis (GAMSTORP and VINNARS 1961) It can be concluded that the changes in neuromuscular transmission in potassium intoxication are related to hyperpotassemia and not to the drop in pH.

The statement in the literature that the pH of the surrounding fluid influences nerve excitability (GRAHAM and LORENTE de Nó 1938) appears to be based on experiments on isolated nerve fibers in which experiments the pH of the surrounding fluid was changed by altering its carbon dioxide concentration (LEHMANN 1937) In our experiments carbon dioxide administration appears to influence neuromuscular transmission more than does the same reduction of pH when produced without increasing carbon dioxide tension.

We are indebted to Dr PHILIP R. DODGE, Dr ROBERT S. SCHWAB and Dr RAYMOND D. ADAMS, Neurology Service, to Dr JOHN D CRAWFORD Children's Medical Service, and to Dr HENRY K. BEECHER and Dr JOHN P BUNKER, Anaesthesia Service for help and encouragement throughout the work and for placing equipment and laboratory facilities at our disposal.

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From the Departments of Neurology and Pediatrics of the Harvard Medical School and the Neurology and Children Medical Services and the Joseph P Kennedy J Memorial Laboratories of the Massachusetts General Hospital and the Anesthesia Laboratory of the Harvard Medical School t the Massachusetts General Hospital, Boston, U S. A.

Studies in Neuromuscular Transmission

II. Influence on Neuromuscular Transmission of Hyperpotassemia

By

INGRID GAMSTORP and ERIK VINCKARS

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Abstract

GAMSTORP I. and E. VINCKARS II *Influence on neuromuscular transmission of hyperpotassemia.* Acta physiol. scand. 1961 53. 151—159 — The effect of acute potassium intoxication on the neuromuscular transmission was studied in rabbits. The sciatic nerve was stimulated through bipolar electrode pairs and the electrical response of the gastrocnemius muscle was recorded. The stimulus necessary to evoke a maximal response, neuromuscular conduction time, nerve conduction time, amplitude and duration of response, refractory and irresponsive periods were measured as was the response to repeated stimulation t a rat of 3 per second. Hyperpotassemia was produced by i. infusion (1/2—1 1/2 ml per min.) of isotonic solution of potassium chloride over 1/2—3 1/2 hours. The observed changes were Prolonged neuromuscular conduction time, decreased amplitude and prolonged duration of response prolonged refractory and irresponsive periods. These findings can be explained by slowing of depolarization and re polarization near at or distal to the neuromuscular junction. They were not caused by the metabolic acidosis nor by the hyponatremia which in some of the experiments accompanied the hyperpotassemia.

Jerry Lewis Fellow of the Muscular Dystrophy Associations of America, Inc. Clinical and Research Fellow in Neurology and Pediatrics at Massachusetts General Hospital and Harvard Medical School Present address Dept. of Pediatrics, Lund, Sweden.

Visiting Lecturer in Anesthesia at Massachusetts General Hospital and Harvard Medical School.

in metabolic acidosis (Gamstorp and Vinnars 1961). It can be concluded that the changes in neuromuscular transmission in potassium intoxication are related to hyperpotasaemia and not to the drop in pH.

The statement in the literature that the pH of the surrounding fluid influences nerve excitability (Graham and Lorente de Nó 1938) appears to be based on experiments on isolated nerve fibers in which experiments the pH of the surrounding fluid was changed by altering its carbon dioxide concentration (Lehmann 1937). In our experiments carbon dioxide administration appears to influence neuromuscular transmission more than does the same reduction of pH when produced without increasing carbon dioxide tension.

We are indebted to Dr. Philip R. Dodge, Dr. Robert S. Schwab and Dr. Raymond D. Adams, Neurology Service, to Dr. John D. Crawford, Children's Medical Service and to Dr. Henry K. Beecher and Dr. John P. Bunker, Anaesthesia Service for help and encouragement throughout the work and for placing equipment and laboratory facilities at our disposal.

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The following parameters were measured (see GAMSTORP and VYSKOČIL 1961) (Fig. 1)

1 The voltage and duration of the stimulus needed to evoke maximal response.

2. Neuromuscular conduction time: the time interval from the beginning of the stimulus applied through the distal electrode pair to the beginning of the response (Fig. 1 c).

3. Nerve conduction time the time interval from the beginning of the stimulus applied through the proximal electrode pair to the beginning of the response minus neuromuscular conduction time (Fig. 1 a—c)

4 The amplitude and duration of the response (Fig 1 b and d)

5. The absolute refractory period the shortest time interval between supramaximal stimuli allowing both t evoke a response (Fig 1)

6 The absolut irresponsive period: the shortest time interval between the responses evoked by 2 supramaximal stimuli (Fig. 1 f)

7 The response to repeated stimulation t rate of 3 per second.

Electrocardiogram was recorded on another channel through a needle electrode in the intercostal muscles for this purpose the sweep speed was 0.2 m per second.

Arterial blood pressure remained t a level above 75 mm Hg The intramuscular temperature varied between 42 and 45° C, not changing more than 0.8° during single experiment.

Potassium intoxication was produced by intravenous infusion of an isotonic potassium chloride solution. During the infusion electrocardiogram and blood pressure were constantly watched. When a drop in the blood pressure or severe hyperpotassemic changes in the electrocardiogram developed the infusion was stopped until improvement occurred. W thout these precautions sudden cardiac arrest occurred unexpectedly leaving no time for recording neuromuscular transmission before convulsions and death.

The pH of the arterial blood was determined using a Beckman pH-meter and the concentration of potassium and sodium in plasma using Beckman flamephotometer One sample was always drawn before the experiment and one at the height of the intoxication during several experiments intermediate samples were obtained.

As can be seen from Table I, substantial drop in the concentration of plasma sodium occurred in several experiments. To exclude the possibility that low concentration of plasma sodium might have influenced the results, another series of experiments was performed on 3 rabbits weighing 2.5 to 2.7 kg The same operativ procedure was done and the same parameters measured as previously described. These rabbits received intravenously 5 dextrose in distilled water containing enough potassium chloride to make its potassium concentration 5 meq/l.

Results

Three rabbits in the main series were excluded because of sudden cardiac arrest with convulsions and death preventing recording during potassium intoxication. The results in the remaining ones are summarized under the headings defined in the section of methods.

A. During potassium ntoxication.

1 The stimulus necessary to evoke a maximal response increased in 2 of 10 exp.

2. Neuromuscular conduction time increased 0 3 to 1 msec in 8 of 10 exp., did not change significantly in one and was not measurable in one.

3 No significant change in nerve conduction time was found.

Table I Blood values during potassium intoxication

Rabbit no.	Weight kg	Duration and amount of infusion	K meq/l	Na meq/l	pH
3	2.3	Before start of infusion	2.9	141	7.25
		60 min., 50 ml	5.8	135	7.32
		165 min., 150 ml	8.5	134	7.32
7	3.2	Before start of infusion	4.2	134	7.30
		45 min., 55 ml	10.7	130	7.25
9	2.2	Before start of infusion	3.1	136	7.30
		150 min., 90 ml	15.3	124	7.05
10	2.1	Before start of infusion	3.4	140	7.33
		45 min., 55 ml	4.3	136	7.25
		165 min., 80 ml	16.7	126	6.80
11	3.2	Before start of infusion	3.7	134	7.33
		60 min 80 ml	7.6	127	7.30
		150 min., 140 ml	9.6	123	7.23
12	1.6	Before start of infusion	3.5	134	7.25
		100 min., 65 ml	7.4	126	7.25
		170 min., 95 ml	12.4	113	7.18
14	3.4	Before start of infusion	4.0	133	7.35
		75 min., 80 ml	7.6	124	7.30
		115 min., 105 ml	7.2	128	7.30
7	3.7	Before start of infusion	2.4		
		35 min., 55 ml	5.6		
31	2.4	Before start of infusion	3.8		
		110 min., 95 ml	5.7		
		210 min., 170 ml	9.2		
90	2.4	Before start of infusion	2.9	137	
		35 min., 50 ml	5.4	139	
		125 min., 100 ml	7.1	133	

As the electrocardiogram showed severe hyperpotassemic changes, the possibility of laboratory error has to be considered.

4 A decrease in the amplitude of the response was noted in all 10 exp. the decrease varied from 1 2 to 14 6 mV A prolongation of the response was also noted in all 10 exp the prolongation varied from 0.5 to 6 msec.

5 An increase in the absolute refractory period was noted in all 10 exp. the increase varied from 0 3 to 1 7 msec.

6 An increase in the absolute irresponsive period was noted in all 10 exp the increase varied from 0 5 to 4 5 msec.

7 Subsequent responses to repeated stimulation at a rate of 3 per second showed no decrease in amplitude as compared with the first one.

Table II. Measurements performed simultaneously with the blood studies listed in Table I

Rabbit no.	Neuro-muscular cond. time msec.	Amplitude of response mV	Duration of response msec.	Abs. refractory period msec.	Abs. irresponsive period msec.
3	1.3	2.0	3.8	1.0	1.7
	1.3	1.5	3.8	0.9	1.7
	2.0	0.8	6.0	2.7	4.0
7	1.2	3.0	4.5	0.9	2.0
	1.6	1.5	5.0	1.5	2.5
9	1.2	7.3	3.5	1.6	2.2
	1.3	3.0	5.0	2.7	4.3
10	1.0	15	3.0	0.8	1.3
	1.0	17	4.0	0.7	2.5
	1.7	12.5	6.0	1.5	4.0
11	1.2	15	4.0	1.0	1.5
	1.5	10	4.0	1.5	3.0
	1.7	0.4	10.0	2.0	3.5
12	1.0	14	4.5	1.0	1.2
	1.1	1.6	6.0	1.0	2.0
	1.6	4.5	6.0	2.0	3.0
14	1.0	13	6.0	0.6	2.0
	1.2	12	6.0	1.4	4.5
	2.0	0.2	9.0	1.7	6.3
27	0.9	17	5.0	0.9	1.0
	1.2	7.0	7.0	1.9	2.7
31	—	17	6.0	0.7	1.6
	1.0	9.0	7.0	0.8	3.0
	1.2	5.5	7.0	1.6	4.5
90	0.9	17	7.0	1.0	2.2
	1.1	14	7.0	0.9	3.7
	1.2	11.5	8.0	1.3	4.4

B During hyponatremia.

1—3 *No significant changes were found.*

4 The amplitude of the response decreased 2 to 4 mV in 2 of 3 exp. and did not change in one. The duration of the response decreased 0.5 msec in 1 exp. increased 1.0 msec in one and did not change in one.

5—7 No significant changes were found.

Blood values and pertinent measurements during potassium intoxication are summarized in Table I and II and during hyponatremia in Table III and IV. Samples of the recordings from 1 exp. are presented in Fig. 1 and 2.

Table I Blood values during potassium intoxication

Rabbit no.	Weight kg	Duration and amount of infusion	K meq/l	Na meq/l	pH
5	2.3	Before start of infusion	2.9	141	7.25
		60 min., 50 ml	5.8	153	7.32
		163 min., 130 ml	8.5	134	7.32
7	3.2	Before start of infusion	4.2	134	7.30
		45 min., 55 ml	10.7	130	7.23
9	2.2	Before start of infusion	3.1	136	7.30
		150 min., 90 ml	15.3	124	7.05
10	2.1	Before start of infusion	3.4	140	7.33
		45 min., 55 ml	4.3	136	7.23
		163 min., 80 ml	16.7	126	6.98
11	3.2	Before start of infusion	3.7	134	7.33
		66 min., 80 ml	7.6	127	7.30
		150 min., 140 ml	9.6	123	7.23
12	1.6	Before start of infusion	3.5	134	7.23
		100 min., 65 ml	7.4	126	7.15
		170 min., 95 ml	12.4	113	7.18
14	3.4	Before start of infusion	4.	133	7.35
		75 min., 80 ml	7.6	124	7.30
		115 min., 105 ml	7.2	128	7.30
27	3.7	Before start of infusion	2.4		
		35 min., 55 ml	6		
31	2.4	Before start of infusion	3.8		
		110 min., 95 ml	5.7		
		210 min., 170 ml	5.2		
90	2.4	Before start of infusion	2.9	157	
		35 min., 50 ml	5.4	139	
		125 min., 100 ml	7.1	153	

As the electrocardiogram showed severe hyperpotassemic changes, the possibility of laboratory error has to be considered.

4. A decrease in the amplitude of the response was noted in all 10 exp.; the decrease varied from 1.2 to 14.6 mV. A prolongation of the response was also noted in all 10 exp.; the prolongation varied from 0.5 to 6 msec.

5. An increase in the absolute refractory period was noted in all 10 exp.; the increase varied from 0.3 to 1.7 msec.

6. An increase in the absolute irresponsive period was noted in all 10 exp.; the increase varied from ... to 4.5 msec.

7. Subsequent responses to repeated stimulation at a rate of 3 per second showed no decrease in amplitude as compared with the first one.

Table II Measurements performed simultaneously with the blood studies listed in Table I

Rabbit no.	Neuro-muscular cond. time msec.	Amplitude of response mV	Duration of response msec.	Abs. refractory period msec.	Abs. irresponsiv. period msec.
3	1.3	2.0	3.8	1.0	1.7
	1.3	1.5	3.8	0.9	1.7
	2.0	0.8	6.0	2.7	4.0
7	1.2	3.0	4.5	0.9	2.0
	1.6	1.5	5.0	1.5	2.5
9	1.2	7.3	3.5	1.6	2.2
	1.3	3.0	5.0	2.7	4.5
10	1.0	13	3.0	0.8	1.3
	1.0	17	4.0	0.7	2.5
	1.7	12.5	6.0	1.5	4.0
11	1.2	13	4.0	1.0	1.5
	1.5	10	4.0	1.5	3.0
	1.7	0.4	10.0	2.0	3.5
12	1.0	14	4.5	1.0	1.2
	1.1	1.6	6.0	1.0	2.0
	1.6	4.5	6.0	2.0	3.0
14	1.0	13	6.0	0.6	2.0
	1.2	12	6.0	1.4	4.5
	2.0	8.2	9.0	1.7	6.5
27	0.9	17	5.0	0.9	1.0
	1.2	7.0	7.0	1.9	2.7
31	—	17	6.0	0.7	1.6
	1.0	9.0	7.0	0.8	3.0
	1.2	5.5	7.0	1.6	4.5
90	0.9	17	7.0	1.0	2.2
	1.1	14	7.0	0.9	3.7
	1.2	11.3	8.0	1.3	4.4

B. During hyponatremia.

1—3 No significant changes were found.

4 The amplitude of the response decreased 2 to 4 mV in 2 of 3 exp and did not change in one. The duration of the response decreased 0.5 msec in 1 exp. increased 1.0 msec in one and did not change in one.

5—7 No significant changes were found.

Blood values and pertinent measurements during potassium intoxication are summarized in Table I and II and during hyponatremia in Table III and IV Samples of the recordings from 1 exp. are presented in Fig 1 and 2.

Table III. Blood values during infusion of dextrose in water

Rabbit no.	Weight kg	Duration and amount of infusion	K meq/l	Na meq/l
87	2.7	Before start of infusion	3.0	139
		75 min., 200 ml	2.9	112
		120 min., 350 ml	3.0	104
88	2.5	Before start of infusion	2.6	138
		30 min., 200 ml	3.0	111
		50 min., 300 ml	3.9	106
89	2.7	Before start of infusion	3.2	143
		15 min., 100 ml	3.7	115
		54 min., 300 ml	3.8	101

Table IV. Measurements performed simultaneously with the blood studies listed in Table III

Rabbit no.	Neuro-muscular cond. time msec.	Amplitude of response mV	Duration of response msec.	Abs. refractory period msec.	Abs. irresponsive period msec.
87	1.4	17	6.0	0.7	2.5
	1.4	17	3.5	0.8	2.1
	1.5	17	5.5	0.6	2.3
88	1.6	14	5.0	1.3	3.7
	1.6	12	6.0	1.5	3.7
	1.3	13	6.0	1.3	3.7
89	1.3	15	5.5	0.9	1.2
	1.3	14	5.5	0.9	1.3
	1.3	11	5.5	1.0	1.6

Discussion

From clinical and experimental studies it is known that hyperpotassemia more often causes cardiac arrest than impaired function of skeletal muscles (Fenn and Marchand 1943, Smillie 1915, Kolff 1950). The same was noted in our experiments. Only when ECG and blood pressure were constantly watched and the speed of the infusion adjusted to changes in them, was it possible to produce severe potassium intoxication without severely impaired circulation. On the other hand the time for the potassium infusion was kept as short as possible. From Table I it is apparent that it never exceeded 3 1/2 hours and usually was about 2. As the uptake of potassium by skeletal muscles in the rabbit is known to be slow, this length of time will allow only a relatively small increase in muscle potassium to occur (Fenn *et al.* 1941).

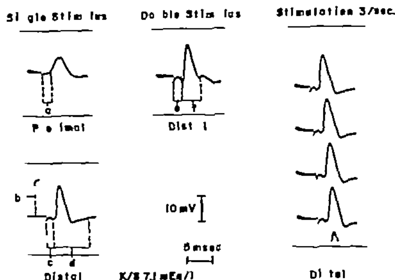


Fig. 2. Rabbit no. 90. Same experiment as in Fig. 1 Recording after intravenous infusion of 100 ml isotonic KCl solution over period of 125 min. Same symbols as in Fig. 1
Lines on the illustrations have been darkened for photographic purposes.

As can be seen from Table I, infusion of potassium chloride caused 2 changes in the blood beside hyperpotassemia. First, a slight to moderate drop in sodium was noted. However th same changes in neuromuscular transmission were found in experiments showing a small and a great decrease in sodium. Furthermore, no change in neuromuscular conduction time, refractory and irresponsive periods were noted during the experiments in which hyponatremia was produced. The low sodium may account for some of the decrease in amplitude of the response but can not explain the other findings observed during potassium intoxication.

Secondly a metabolic acidosis was often produced. However th same changes in neuromuscular transmission were noted in experiments showing severe acidosis as in those showing none. Furthermore, although it has been observed that neuromuscular conduction time is slightly prolonged in severe respiratory acidosis, neither this nor other changes in neuromuscular transmission were noted during severe metabolic acidosis (GAMSTORP and VINCKARD 1961) The changes in neuromuscular transmission found during infusion of potassium chloride are thus related to the increase of potassium and not to a decrease n sodium and pH.

When directly applied to the muscle fiber or given in close arterial injection" potassium has a depolarizing effect (BUCHTHAL and LINDHARD 1939) If the close arterial injection" was given during intermittent supramaximal nerve stimulation the mechanical response of the muscle increased in amplitude

while the electrical one decreased (BROWN 1937). On recording mechanically and electrically the muscle response to nerve stimulation in rats during hyperpotassemia produced by an intraperitoneal injection of potassium chloride, WALKER (1948) was able to confirm both these observations concerning the amplitude. Furthermore, he found an increase in the interval between stimulus and response and in the duration of the response. He concluded that an excess of potassium delays both depolarization and repolarization and increases the duration of membrane breakdown.

Our experiments thus confirm to a large extent WALKER's observations and his interpretations. Neuromuscular conduction time was found to be increased while excitability and conduction velocity in the nerve itself were unchanged. This means that the delay occurs near, at or distal to the neuromuscular junction. A prolongation of the response may be caused by a slower conduction of the potential over the muscle fiber, by an increased asynchrony of the individual muscle fibers within a motor unit or by a slower repolarization. Hyperpotassemia of this degree has been shown not to decrease conduction velocity over the electrically stimulated muscle fiber (BUCHTHAL *et al.* 1958) and for this reason the first suggestion is improbable. An increased asynchrony will cause both lower amplitude and longer duration and can also explain the enhanced mechanical response found by others (BROWN 1937, BROWN and EULER 1938, WALKER 1948). Only a prolonged contraction time, i. e. a slower repolarization, however, can account for our findings of prolonged refractory and irresponsive periods. Thus, all of our findings in potassium intoxication can best be explained by a slower depolarization and repolarization near, at or distal to the neuromuscular junction.

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From the Anesthesia Laboratory of the Harvard Medical School at the Massachusetts General Hospital and the Departments of Neurology and Pediatrics of the Harvard Medical School and the Neurology and Children's Medical Services and the Joseph P. Kennedy Jr. Memorial Laboratories of the Massachusetts General Hospital, Boston, U. S. A.

Studies in Neuromuscular Transmission

III. Influence of Changes in Blood pH and Carbon Dioxide Tension on the Effect of Tubocurarine and Dimethyl Tubocurarine

By

INGRID GAMSTORP[2] and ERIK VINNARS[1]

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Abstract

GAMSTORP, I. and E. VINNARS. III. *Influence of changes in blood pH and carbon dioxide tension on the effect of tubocurarine and dimethyl tubocurarine.* Acta physiol. scand. 1961. *53.* 160—173. — The influence of changes in pH and the partial pressure of carbon dioxide in arterial blood on the neuromuscular blocking effect of a single dose of tubocurarine or dimethyl tubocurarine was investigated in rabbits. The sciatic nerve was stimulated supramaximally at a rate of 3 per second and the electrical response of the gastrocnemius muscle was recorded; the amplitude of the first and fourth response was measured. During the period of spontaneously subsiding block after a single injection of the blocking agent, a shift in the arterial pH was produced either with or without a change in carbon dioxide tension. An increase of the block during this period was always considered significant but a decrease of the block only if it was followed by a spontaneous increase. The action of tubocurarine increased during acidosis and diminished during alkalosis. This effect was greater during metabolic alkalosis and acidosis, corresponding to a more rapid shift in pH, than during respiratory alkalosis and acidosis, but no qualitative difference was observed. The effect of a shift in pH is explained by changes in the potency of tubocurarine. The action of dimethyl tubocurarine was not influenced by changes in pH or carbon dioxide tension.

[1] Visiting Lecturer in Anesthesia at Massachusetts General Hospital and Harvard Medical School.

[2] Jerry Lewis Fellow of the Muscular Dystrophy Association of America, Inc. Clinical and Research Fellow in Neurology and Pediatrics at Massachusetts General Hospital and Harvard Medical School. Present address: Dept. of Pediatrics, Lund, Sweden.

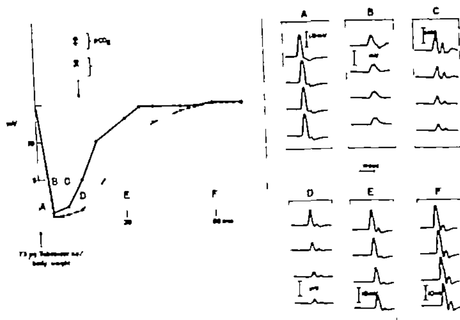


Fig. 1 Rabbit no. 61 The curve shows the amplitude of first response (unbroken line) and of fourth response (broken line) recorded electrically in left gastrocnemius muscle after supramaximal stimulation of sciatic nerve at rate of 3 per second. Examples of tracings are given to the right in the figure letters indicate when the recordings were made.

Changes in carbon dioxide tension and blood pH are known to influence the action of neuromuscular blocking agents in clinical anesthesia (DUNDEE 1952, SCURR 1954 GRAY and FENTON 1954) PAYNE (1958, 1959) and JOHANSEN and OSGOOD (1960) have experimentally confirmed in cats the enhancing effect of carbon dioxide on the action of tubocurarine. The influence of carbon dioxide on the block caused by dimethyl tubocurarine was found to be in the opposite direction. Recently PAYNE (1960) found that the effect of both tubocurarine and dimethyl tubocurarine increased during metabolic alkalosis and decreased during metabolic acidosis. He concluded that carbon dioxide has a specific effect on tubocurarine differing from that of changes in pH. PAYNE's results and interpretation contrast with the observation of KALOW (1954) who found that a reversible change in the ionization of tubocurarine followed variations in pH and increased its blocking effect on the isolated nerve muscle preparation when pH decreased. The following experiments were designed to obtain further information about variations in the sensitivity of the intact animal to tubocurarine and dimethyl tubocurarine.

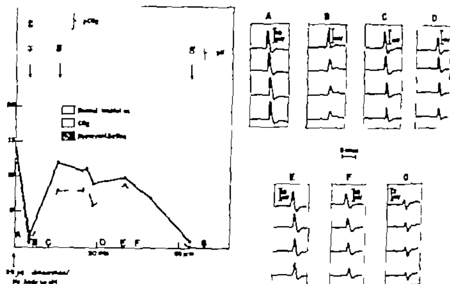


Fig. 2 Rabbit no. 22. The curve shows the amplitude of first response (unbroken line) and of fourth response (broken line) recorded electrically in left gastrocnemius muscle after supramaximal stimulation of sciatic nerve at a rate of 3 per second. Type of ventilation, blood pH and pCO are given above the curve. Examples of tracings are given to the right in the figure; letters indicate when the recordings were made.

Material and Methods

Forty-one albino rabbits weighing 2 to 4 kg were used. Twenty-one (group I) received tubocurarine and 20 (group II) dimethyl tubocurarine. For the operative procedure pentobarbital sodium (28 mg per kg) was given i.v. about 2 hours before the experiment started. Procaine chloride locally was the only other anesthetic administered. The right femoral artery and vein were catheterized. A tracheal cannula was inserted through a tracheostomy. The left sciatic nerve was exposed and a pair of stainless steel electrodes was applied to it. Proximal to the electrode pair the nerve was crushed and ligated. The divided gluteal muscles were sutured and the skin closed.

The stimulus was a square wave pulse of 10 to 50 μsec duration and 15 to 150 V strength. It was maintained supramaximal throughout the experiment. The electrical response of the left gastrocnemius muscle was recorded through a concentric needle electrode on a Disa electromyograph. The permanent recording of interrupted sweeps was made on photographic paper with a sweep speed of 2 m per second. Stimulation at a rate of 3 per second for about 2 sec was used to demonstrate the effect of tubocurarine on the neuromuscular transmission: the amplitude of the first and fourth response was measured. At the start of the experiment the needle electrode was adjusted until a simple response was obtained with the highest possible amplitude and a sharp initial deflection. The electrode was then fixed in this position.

During curarization artificial respiration was given with a positive-negative pressure pump. Frequency and tidal volume were adjusted to maintain normal blood pH and carbon dioxide tension except when alkalosis or acidosis was deliberately produced.

In order to maintain normal blood volume dextran was infused i.v. in amounts roughly corresponding to the blood loss. Arterial blood pressure was measured con-

Table I. Blood values in respiratory alkalosis and acidosis

Rabbit no.	Tubo-curarine dose mg/kg	Experimental condition	pH	pCO_2 mm Hg	CO_2 content meq/l	K meq/l
28	0.14	Normal ventilation	7.39	33	20.7	3.5
		Hyperventilation	7.58	18.6	17.7	3.5
		10 % CO_2	7.00	102.0	37.3	3.6
		20 % CO_2	6.87	111	24.9	
30	0.14	Normal ventilation	7.51	22	17.5	3.3
		Hyperventilation	7.79	10	14.2	3.4
		10 % CO_2	7.10	76	25.3	3.4
32	0.15	Normal ventilation	7.41	29	18.8	
		Hyperventilation	7.62	14.5	14.7	2.8
		10 % CO_2	7.41	37	24.1	4.1
		20 % CO	7.02			3.9
33	0.14	Hyperventilation	7.68	10	11.5	2.4
		Normal ventilation	7.43	20	13.6	2.1
		20 % CO	6.84	120	27.4	2.4
38	0.14	Hyperventilation	7.72	13.3	16.9	2.7
		Normal ventilation	7.46	24.2	17.4	2.6
		10 % CO	7.05	104	29.3	2.4
41	0.18	Hyperventilation	7.53	17.4	14.8	4.2
		Normal ventilation	7.29	30.2	15.2	3.4
		10 % CO_2	7.05	74	22.4	3.6

stantly and maintained at level bove 70 mm Hg. The intramuscular temperature varied between 41 and 45 C, not changing mor than 1.5 during ingle experiment.

Group I. An initial recording was made t the start of the experiment. A single dose of tubocurarine (0.14 to 0.18 mg per kg) was then given i. a. and new recording was made 5 min later. During this time entilation was maintained normal. In 5 rabbits (group I A) the effect of tubocurarine alone was followed until it disappeared, recordings being made every 5 min.

I 9 rabbits (group I B) alkalosis was induced by increasing tidal volume 2.5 to 3 times directly following the recording made 5 min after the injection of tubocurarine. Hyperventilation was continued for 10 min followed by 10 min of normal ventilation, after which acidosis was produced by administration of 5 (this concentration was tested in only 4 exp.) 10 and 20 % carbon dioxide in oxygen, each concentration for a period of 10 min.

A the end of each 10 min period recording was regularly made and arterial blood was usually drawn. The pH of the arterial blood was determined using a Beckman pH meter and carbon dioxide content was measured on the same sample by the method of an Sl ke the partial pressure of carbon dioxide in arterial blood was calculated from Singer Hastings nomogram. The concentration of potassium in plasma was determined on Beckman flamephotometer

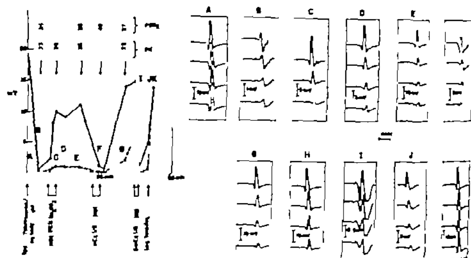


Fig. 3 Rabbit no. 47 The curve show the amplitude of first response (unbroken line) and of fourth response (broken line), recorded electrically in left gastrocnemius muscle after supramaximal stimulation of sciatic nerve at a rate of 3 per second. Type and amount of injection is given below the curve, blood pH and pCO above it. Examples of tracings are given to the right In the figur letters indicate when the recordings were made.

In 7 rabbits (group I C) alkalosis was produced by i.v. injection of 6 to 16 meq 0.5 N sodium carbonate solution over 2 to 3 min. The infusion started directly following the recording made 5 min after the injection of tubocurarine. Recordings were made within 1 min after the injection and bout 5 to 10 min later Within 15 min after the injection of sodium carbonate, metabolic acidosis was produced by v i jection of 7 to 9 meq 0.5 N hydrochloric acid over 2 to 5 min. During the injection the tidal volume was increased 10 to 20 % to maintain as normal a carbon dioxide tension as possible. Recordings were made within 1 min after the injection and 5 mi after and, if the rabbit survived long enough, 15 min later Samples of blood for pH carbon dioxide content and plasma potassium were obtained simultaneously with the electrical ecordings

Group II An initial recording was made t the start of the experiment A single dose of dimethyl tubocurarine (10 to 44 μg per kg) was given i a. and a new recording was made 5 min later I 4 rabbits (group II A) the effect of dimethyl tubocurarine alone was followed until it disappeared, recordings being made every 5 min.

I 6 rabbits (group II B) respiratory alkalosis and acidosis were induced in the same way as described for group I B. Four rabbits were first exposed to hypervi entilation followed by normal entilation and finally administration of carbon dioxide In 2 rabbits the opposite order was used Two rabbits were exposed twice t nhalation of 20 carbon dioxide At the end of each 10 min period a recording was made and usually arterial blood was draw for determination of pH, carbon dioxide ontent and plasma potassium.

In 10 rabbits (group II C metabolic alkalosis and acidosis were induced in the same way as described for group I C. I 6 rabbits alkalosis was first produced followed by acidosis, in 4 the opposit order was used.

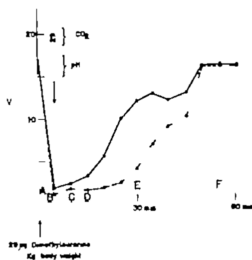
As alkalosis and acidosis in groups B and C were produced during the phase of rapidly subsiding neuromuscular block, any enhancement of the block was considered significant but decrease of the block only if it was followed by a spontaneous increase

Table II Blood values in metabolic alkalosis and acidosis

Rabbit no.	Tubo-curarine dose mg/kg	Experimental condition	pH	pCO_2 mm Hg	CO_2 content meq/l	K meq/l
47	0.16	Initial value	7.33	34.2	18.9	
		After 7 meq Na_2CO	7.50	31.8	23.2	2.2
		10 min. after injection	7.38	35.8	22.0	
		After 7 meq HCl	7.12	40.0	14.0	3.2
		10 min. after injection	7.22	37.3	16.2	
48	0.18	Initial value	7.20			
		After 9 meq Na_2CO	7.62			3.2
		8 min. after injection	7.42			
		After 9 meq HCl	7.19			3.7
		10 min. after injection	7.30			
59	0.16	Initial value	7.44	34	23	2.8
		After 16 meq Na_2CO	7.90		58	5.7
		6 min. after injection	7.70			
		After 8 meq HCl	7.01			
60	0.16	Initial value	7.61	29	30	3.1
		After 16 meq Na_2CO_3	8.00		60	5.1
		4 min. after injection	7.59			
		After 7 meq HCl	6.90	50	9	5.0
63	0.17	Initial value	7.43	35	25	2.8
		After 6 meq Na_2CO	7.85	26	46	2.7
		5 min. after injection	7.68			
		After 9 meq HCl	6.96	44	10	
64	0.17	Initial value	7.49	33	26	2.5
		After 8 meq Na_2CO_3	7.76	28	42	2.7
		5 min. after injection	7.61			
		After 8 meq HCl	7.05	47	14	4.7
		6 min. after injection	7.37			
		After another 3.5 meq HCl	6.92	43	9	
65	0.17	Initial value	7.51	28	23	2.9
		After 8 meq Na_2CO	7.74	26	33	3.0
		5 min. after injection	7.67			
		After 8 meq HCl	7.18	28	11	2.8
		4 min. after injection	7.34			
		After another 7 meq HCl	6.98			

Results

Group I A. Fig. 1 illustrates the curve for spontaneous recovery of neuromuscular transmission after a single injection of tubocurarine in 1 exp. The pattern in the other 4 was similar with the maximal block developing within 5 min after the injection of tubocurarine. Thereafter the block steadily diminished



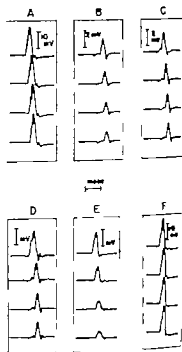


Fig 4. Rabbit no. 80. Same as in Fig 1

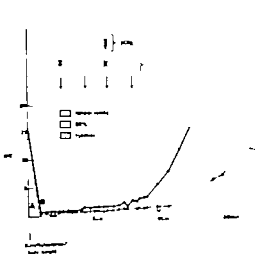


Fig 5 Rabbit no. 83. Same in Fig

Table III. Blood values in respiratory alkalosis and acidosis

Rabbit no.	Dimethyl tubocurarine µg/kg	Experimental condition	pH	pCO mm Hg	CO_2 content meq/l
78	37	Normal ventilation	7.60	25	24
		Hyperventilation	7.76	26	37
		Normal ventilation	7.43	32	22
		5 % CO	7.17	>80	33
		10 CO	7.10	>80	34
		20 „ CO	6.96	>80	36
79	30	Normal ventilation	7.33	23.2	20
		20 % CO_2	6.93		
		10 % CO_2	7.10	80.5	26.9
		5 % CO_2	7.22	60.5	26.0
		Normal ventilation	7.49	26.6	20.5
		Hyperventilation	7.79		
84	30	Hyperventilation	7.72	12.4	15.7
		Normal ventilation	7.44	25.7	20.0
		5 % CO	7.23	50	22.9
		10 CO	7.14		
		20 „ CO	6.98		
85	33	20 CO	7.00	>80	23
		Normal ventilation	7.52	23	19
		Hyperventilation	7.78	<10	12
		20 CO_2	6.98		
105	12	Hyperventilation	7.66	11.8	12.9
		Normal ventilation	7.48	22	16.7
		20 CO	6.92	>120	28.6
106	12	Hyperventilation	7.71		
		Normal ventilation	7.32	18.5	13.2
		20 „ CO	7.02		

and had disappeared completely 30 to 70 min after the injection. These findings are in agreement with the results of other examiners (KALOW 1959). It is particularly important to stress that a spontaneous increase in the block was never observed during the recovery period.

Group I B. In this group 3 rabbits were excluded because of severe anoxia and subsequent metabolic acidosis. 6 exp. thus remain for analysis. In all of them the effect of tubocurarine diminished during hyperventilation. However this occurred during the period of spontaneously subsiding block. The effect of hyperventilation was therefore not considered significant unless an increase in the block was observed during the period of normal ventilation following hyperventilation. This type of curve was found in 4 exp., while in 2 (rabbit

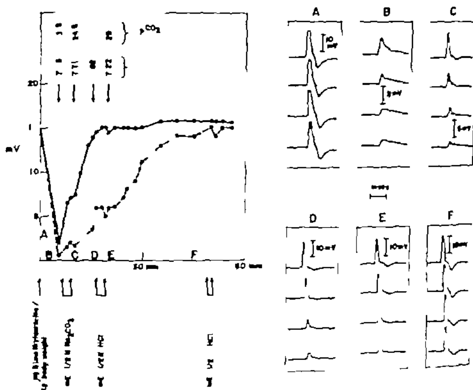


Fig 6. Rabbit no. 77 Same as in Fig 3.

no 26 rabbit no 30) the block continued to decrease during normal ventilation.

Administration of 5 % carbon dioxide did not influence the block significantly An increase of the block was noted after either 10 or 20 % carbon dioxide in all 6 exp and in 4 of 6 after both (rabbit no 41 showed no change after 10 rabbit no 30 not after 20 carbon dioxide) These changes are significant as they occurred during a period when rapidly decreasing block could be expected In 3 exp. Tensilon (0 02 to 0 03 mg) reversed the block during continued administration of carbon dioxide, while in another normal ventilation had the same effect. The result of a typical experiment is given in Fig 2. Table I shows that respiratory alkalosis and acidosis were produced without significant change in the concentration of potassium in the plasma.

Group I C. The results were identical in all 7 rabbits in which metabolic alkalosis and acidosis were induced during curarization. The block decreased steadily during the injection of sodium carbonate and had almost disappeared 1 min after the end of the injection at which time the pH of the blood had increased. When the pH dropped the block again increased A marked en-

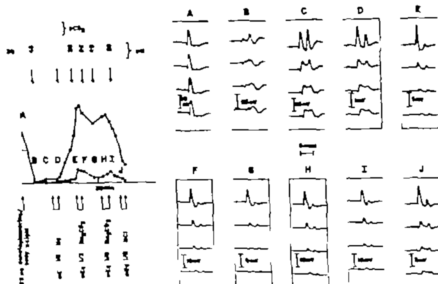


Fig. 7 Rabbit no. 76. Same as in Fig. 3
Lines on illustrations have been darkened for photographic purpose.

hancement of the block occurred when the pH of the blood was lowered by i. v. injection of hydrochloric acid. When the pH rose spontaneously the block again diminished. In 4 exp. a second injection of hydrochloric acid about 10 min later enhanced the block once more. In 2 exp. 0.1 mg of Tensilon given immediately after hydrochloric acid reversed the block, in another experiment sodium carbonate had the same effect.

The result of a typical experiment is given in Fig. 3. Table II summarizes the results of analysis of the blood samples showing that a marked change in pH was produced with only a small shift in carbon dioxide tension and no significant change in the concentration of potassium in the plasma.

Group II A. Fig. 4 illustrates the curve for spontaneous recovery of neuromuscular transmission after a single dose of dimethyl tubocurarine. The pattern in the other 3 exp. in this group was similar with the maximal block developing within 5 min. Thereafter the block steadily diminished and had disappeared *completely 50 to 90 min later. The slight spontaneous increase once during the* period after the first 5 min seen in Fig. 4 was not observed in the other experiments. The blocking action of dimethyl tubocurarine was found to be stronger than that of tubocurarine. Its effect was also less uniform and the block varied greatly in severity and duration in different rabbits.

Group II B. The relative order between alkalosis and acidosis did not affect the results. No influence of respiratory alkalosis or acidosis on the block was

Table IV Blood values in metabolic alkalosis and acidosis

Rabbit no.	Dimethyl tubocurarine µg/kg	Experimental condition	pH	pCO_2 mm Hg	CO_2 content meq/l
76	29	Initial value	7.45	31	22
		After 8 meq HCl	7.01	41	14
		7 min. after injection	7.20		
		After 8 meq Na_2CO_3	7.58		
		5 min. after injection	7.40		
		After 8 meq Na_2CO_3	8.20		
77	33	Initial value	7.45	23.5	16.4
		After 9 meq Na_2CO	7.71	24.8	30.7
		7 min. after injection	7.62		
		After 8 meq HCl	7.22	25	10.9
81	33	Initial value	7.49	26.6	20.5
		After 8 meq HCl	6.88	41.0	8.7
		7 min. after injection	7.24		
		After 8 meq Na_2CO_3	7.58	27	23.9
		7 min. after injection	7.42		
82	29	Initial value	7.49	31	24
		After 8 meq Na_2CO	7.75	24	33
		6 min. after injection	7.61		
		After 7.5 meq HCl	7.12	38	14
101	20	Initial value	7.32	48	23.8
		After 8 meq Na_2CO_3	7.80	30	46.7
		5 min. after injection	7.51		
		After 8 meq HCl	7.20	48	19.9
102	15	Initial value	7.60	19	19
		After 9 meq Na_2CO	7.93	20	44
		6 min. after injection	7.75		
		After 5.5 meq HCl	6.88	34	7
103	23	Initial value	7.45	29	20
		After 9 meq Na_2CO_3	7.78	27	40
		6 min. after injection	7.60		
107	12	Initial value	7.52	22	18
		After 7.5 meq Na_2CO_3	7.75	24	33
		8 min. after injection	7.69		
		After 9 meq HCl	6.80	44	8
		10 min. after injection	7.20		
108	36	Initial value	7.43	26	18
		After 9 meq *HCl*	6.88	41	9
		8 min. after injection	7.18		
		23 min. after injection	7.28		
	44	Another dose of dimethyl tubocurarine			
		After 7 meq HCl	6.87		
109	23	Initial value	7.58	18.7	17.5
		After 4.5 meq HCl	7.21	15.5	6.6
		7 min. after injection	7.22		

noted in 5 of 6 rabbits. In 2 rabbits 20 % carbon dioxide was administered twice without effect. In a single experiment (rabbit no 84) administration of 20 % carbon dioxide was accompanied by an increase in the effect of dimethyl tubocurarine in none was it influenced by hyperventilation.

Fig 5 illustrates the result in one of the negative experiments. That pure or almost pure respiratory alkalosis and acidosis had been produced was confirmed by analysis of the blood samples. The results are listed in Table III

Group II C The relative order between alkalosis and acidosis did not affect the results. No influence on the block of metabolic alkalosis or acidosis was noted in 6 of 10 rabbits. The block diminished in 3 rabbits (no 76 101 103) during alkalosis to increase again when the pH of the arterial blood dropped spontaneously This change, which could be produced twice in 1 rabbit (no 76) was weak and approached the marked effect on a block produced by tubocurarine in a single experiment (rabbit no 103) A slight enhancement of the block was noted during metabolic acidosis in 3 rabbits (no. 76 103 107) in none approaching the marked effect on a block produced by tubocurarine.

Fig 6 illustrates the result in a negative experiment and Fig 7 in an experiment showing a weak influence by metabolic alkalosis and acidosis. Table IV summarizes the results of th analysis of the blood samples showing that a marked change in pH was produced with only a small shift in carbon dioxide tension.

Discussion

I The results of our experiments with metabolic alkalosis and acidosis indicate that the blocking action of tubocurarine varies with changes in the pH of the blood, increasing at a low pH and decreasing at a high pH. The shift in the block induced by respiratory alkalosis and acidosis was of the same type but of less magnitude. This can be explained by a smaller (only during alkalosis, see Table I and II) and slower (both during alkalosis and acidosis) change in blood pH in metabolic alkalosis and acidosis a shift in the pH of the blood was ind ced over 2—3 min, whereas it required 10 min to occur during respiratory alkalosis and acidosis. The time factor is important as the changes in pH were prod ced during the phase of rapidly decreasing block after a single injection of tubocurarine. Furthermore, the respiratory experiments were extended over longer period as 2 or 3 different concentrations of carbon dioxide were tested. The reaction to a shift in the pH of the blood is thus qualitati ely th same whether or not this shift is induced by changes in carbon dioxide tension.

The effect of changes in carbon dioxid and blood pH (GAMSTORP and VINKARS, 1961) on th neuromuscular transmission have previously been shown to be minimal and cannot account for the altered sensitivity to tubocurarine. Release of epinephrine known to influence the effect of curare

(NAESS and SIRNES 1953) could not explain the decrease in block during alkalosis. Furthermore, the increased curare block during acidosis was reversible either by administration of Tensilon or by raising the pH of the blood. It can thus be concluded that a shift in the pH of the blood influences the effect of tubocurarine principally by changing the apparent potency of the drug

The results of PAYNE's (1958) experiments on the sensitivity to tubocurarine during respiratory acidosis in cats are similar to ours. Our findings during respiratory alkalosis correspond to the clinical observation by DUNDEE (1952) that hyperventilation increases the dose of tubocurarine required for abdominal surgery during the first hour of the operation.

Our results during metabolic alkalosis and acidosis are, however contrary to PAYNE's (1960) One explanation might be that different species were used, but non-depolarizing blocking agents are known to act in essentially the same way in both these species (FOLDES 1959) The fact that PAYNE recorded the mechanical and we the electrical response might play a role, as metabolic changes could conceivably influence the contractility of the muscle which would not be reflected in the electrical response. This could scarcely be the whole explanation since PAYNE (1960) found a variable influence of metabolic alkalosis and acidosis on the mechanical response in the uncurarized animal. Of greater importance may be that by using stronger solutions and faster injection rates we produced a more rap d shift in the pH of the blood. This allowed us to measure changes in the effect of tubocurarine during the phase of rapidly diminishing block after a single injection of the drug Finally PAYNE does not state whether he used a supramaximal stimulus and maintained is so throughout the experiment. He produced metabolic alkalosis by infusing sodium bicarbonate thereby also raising the carbon dioxide tension which will increase the stimulus necessary to evoke a maximal response (GAMSTORP and VINNARS 1961) If the applied stimulus was not above the raised level necessary for evoking a maximal response this mechanism might be responsible for an apparent increase in the block.

II As dimethyl tubocurarine is manufactured from tubocurarine, a ariable trace of the latter drug may conceivably remain in the former thereby explaining its variable potency In most of the experiments changes in blood pH did not influence the block occurring after injection of dimethyl tubocurarine any influence found was always n the same d rection but much weaker than on the block produced by tubocurarine. A small, variable amount of tubocurarin n dimethyltubocurarine could expla n these findings thus making it unnecessary to assume any influence by changes in blood pH on the block produced by d methyl tubocurarine Changes in the partial pressure of carbon dioxide in arterial blood d d not influence the block.

Our results are n disagreement with those obtained by PAYNE (1959 1960) who found the block produced by dimethyl tubocurarine to increase during alkalosis and decrease during acidosis. We are unable to explain this discrepancy

Tubocurarine and dimethyl tubocurarine are both non-depolarizing blocking agents with principally the same effect at the neuromuscular junction. Yet only the block produced by tubocurarine is influenced by changes in blood pH. The difference is well explained by KALOW's (1954) observation that the ionization of tubocurarine but not of dimethyl tubocurarine changes with the pH of the solution.

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From the Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden

Restoration of Vasoconstrictor Effects in Reserpinized Cats[1]

By

SUNE ROSELL and GÖRAN SEDVALL

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Abstract

ROSELL, S. and G. SEDVALL, *Restoration of vasoconstrictor effects in reserpinized cats.* Acta physiol. scand. 1961. 53. 174—184. — Reserpine completely abolishes the effects of vasoconstrictor nerve stimulation (BURN and RAND 1958, ROSELL and ROSÉN 1961). Dopa, dopamine, noradrenaline and adrenaline infused intra-arterially 20—24 hours after the administration of reserpine partially restored the responses to stimulation of the sympathetic chain. The responsiveness persisted for at least one hour after termination of dopa, dopamine and noradrenaline infusion. With adrenaline, however, vasoconstrictor responses to sympathetic chain stimulation persisted for only a short period after termination of the infusion. The threshold dose for dopa was 2 mg, for dopamine and noradrenaline 25 μg, and for adrenaline 100 μg.

The data are considered to support the hypothesis of BURN and RAND (1960) that the adrenergic nerve terminals or adjacent tissue in reserpinized animals have the ability to store infused dopamine and noradrenaline. Adrenaline seems to be stored to a much lesser extent. Dopamine is presumably converted to noradrenaline which is then released when the sympathetic chain is stimulated. The weak restoration of vasoconstrictor responses following infusion of dopa is of interest. The degree of restoration of the vasoconstrictor effects following infusion of noradrenaline is presumably related to the time elapsed between the administration of reserpine and the infusion.

[1] A preliminary report of this work was presented at the Second Scandinavian Summer Meeting of Biochemistry, Medical Chemistry, Pharmacology and Physiology, Joint Meeting with the Biochemical Society, Turku, in August, 1959.

Reserpine blocks the effects of sympathetic vasoconstrictor nerve activity apparently due to a depletion of the adrenergic transmitter substance at the nerve terminals (BERTLER, CARLSSON and ROSENGREN 1956, MUSCHOLL and VOGT 1958 BURN and RAND 1958) On the other hand, the cholinergic vasodilator nerves appear to be functionally intact in reserpinized animals. After reserpine treatment, therefore, stimulation of the sympathetic chain produces vasodilatation in the skeletal muscle vessels (ROSELL and ROSÉN 1961)

In the circulatory studies on reserpinized cats reported by ROSELL and ROSÉN (1961) the low blood pressure was a disturbing factor In order to raise the blood pressure to a more adequate level intravenous infusion of noradrenaline was tried. This unexpectedly led to reappearance of signs of vasoconstrictor activity In the hope of accounting for this restoration of vasoconstrictor effects a series of experiments was instituted, and it was subsequently disclosed that the vasoconstrictor effects could be partially restored by intra-arterial infusion of adrenaline, noradrenaline, dopamine and dopa. While the work was in progress BURN and RAND (1958, 1960) reported that infusion of noradrenaline restored the vasoconstrictor effects in the perfused hind leg of the reserpinized dog They noted, furthermore, that the responses to stimulation of fibers to the nictitating membrane and to the iris were restored by infusion of dopamine or dopa.

The present study deals exclusively with the effects of dopa, dopamine, noradrenaline and adrenaline on the vasoconstrictor responses to sympathetic chain stimulation in skeletal muscle vessels of the reserpinized cat.

Methods

The experiments were performed on cats weighing 2.0 to 4.4 kg under urethane (600—1,200 mg/kg) anesthesia. The trachea was cannulated. The arterial pressure was recorded from one of the carotids by mercury manometer or Statham pressure transducer (P 23 AA) Reserpine (5 mg/kg) was administered subcutaneously 20—24 hours before the experiments.

Blood flow was measured by cannulating the femoral artery and directing the blood through silicone filled drop chamber operating an ordinate writer (LINDGREN 1958) The blood re-entered the hind leg via the cannulated distal stump of the same artery In order to measure only muscle blood flow the hind leg was skinned, and tight ligature around the ankle isolated the paw from the circulation. In most experiments blood flow was recorded from each skinned hind limb separately In one limb intra-arterial infusions were made through a side arm of the arterial drop chamber The other limb served as control. To prevent clotting, heparin (25 mg/kg) was given i. Dextran was administered i. as required to compensate for blood loss.

In those experiments in which blood flow was measured in both hind limbs, both sympathetic chains were isolated via the anterior approach and subsequently cut. When only one hind limb was prepared for blood flow measurements, the ipsilateral sympathetic chain was isolated and cut. A bipolar silver electrode was applied to the distal part of the transected sympathetic chain at the level of L_4—L_5. When both chains were stimulated, they were placed on the same electrode. Supramaximal stimulation voltage produced by a Grass Model S4 stimulator was used throughout.

Rectal temperature was maintained at 36—37° C by means of a heating lamp.

Usually the measurements were recorded on a kymograph. In some experiments a Grass Polygraph was used as a recording instrument.

The vascular resistance was calculated as the ratio of arterial pressure to flow.

Substances used.

Reserpine (Serpedin, Pharmacia) 2.5

Dopa (L (—) Dihydroxy-phenylalanin, Hoffmann-La Roche)

Dopamine (3-Hydroxy-tyramin-hydrochlorid, Hoffmann-La Roche).

Adrenaline (L-adrenaline, ACO).

Noradrenaline (Noradrenalin-Conc., Byk-Gulden, L-noradrenalin. bitartr.).

Dextran (Macrodex, Pharmacia)

Heparin (Heparin, Vitrum) 5

Atropine (Atropin. sulfas, Sw. Ph. XI).

The drugs for intra-arterial infusions were dissolved in 0.9 per cent saline. The following concentrations were used

Dopa 1 mg/ml Dopamine, Noradrenaline and Adrenaline 10 μg/ml.

The catechol amines are expressed as bases in data concerning dosage given.

Results

1 *Intra-arterial Infusion of Noradrenaline*

In 18 reserpinized cats noradrenaline was infused i. a. The total dose varied between 15—100 μg and the infusion rate between 1—2 μg/min. After infusion of a minimum of 25 μg noradrenaline, electrical stimulation of the sympathetic chain induced vasoconstrictor responses. Consistent results were obtained in all experiments. Fig. 1 illustrates a typical experiment. Numbers 1 2 and 3 show the vasodilator responses in a reserpinized cat at different stimulation frequencies. The effects could be blocked by atropine given i. v. (4 5 6). After atropine, stimulation elicited no consistent change in blood flow neither vasodilatation nor vasoconstriction was apparent, presumably because reserpine had completely abolished the vasoconstrictor responses and atropine the vasodilator responses (Rosell and Rosén 1951). Between 6 and 7 50 μg of noradrenaline was infused into the left femoral artery. It will be seen (7 8 and 9) that 10 min after completion of the infusion, stimulation of the sympathetic chain evoked pronounced vasoconstrictor effects in the left hind limb. Numbers 10 11 and 12 indicate the vasoconstrictor responses 60 min after termination of the noradrenaline infusion. Vasoconstriction again occurred in the left hind limb, although the effects this time were less pronounced. After 120 min, however, the blood flow showed no more than a tendency to decrease on sympathetic chain stimulation. Between numbers 15 and 16 noradrenaline (25 μg) was again infused into the left femoral artery. The series of stimuli of the sympathetic chain (16 17 18) initiated 10 min after termination of the infusion, show that it was once more possible to elicit vasoconstrictor effects.

In Fig. 2 the stimulation frequency-response curves following infusion of 100 μg of noradrenaline in reserpinized cats are compared with equivalent

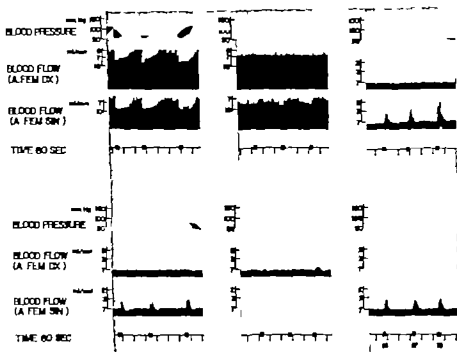


Fig. 1 Cat 3.0 kg. Reserpine 5 mg/kg. Urethane 600 mg/kg. Vasomotor responses in skeletal muscle to stimulation of both sympathetic chains at L—L before and after infusion of noradrenaline into the left femoral artery
1 Stimulation, 2.5 V 8 imp/sec 15 sec.
2. 2.5 V 10 imp/sec 15 sec.
3. 2.5 V 20 imp/sec 15 sec.
Between 3 and 4 0.5 mg/kg atropine intravenously
4. = 1 5. = 2, 6. = 3.
Between 6 and 7 30 µg noradrenaline infused for 50 min.
7 = 1 10 min after infusion had ceased.
8. = 2, 9 = 3.
10. = 1 60 min after infusion had ceased.
11 = 2, 12. = 3.
13. = 1 120 min after infusion had ceased.
14 = 2, 15. = 3.
Between 15 and 16, 25 µg noradrenaline infused for 25 min.
16. = 1 10 min after infusion had ceased.
17 = 2, 18. = 3.
Note the vasoconstrictor responses after infusion of noradrenaline.

curves from "normal" cats. Two factors influenced the observed stimulation responses (1) the elapsed time between the end of the infusion and the stimulation and (2) preceding stimulation. Each stimulation appeared to reduce the available amount of transmitter substance to such a degree that the response to the next stimulation was less than predicted. In order to gauge the effects of these two factors on the stimulation responses, two different sequences of

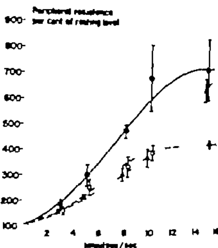


Fig. 2. *Peripheral resistance in percent of resting level during stimulation of the sympathetic chain after intra-arterial infusion of 100 µg noradrenaline in reserpinized cats.*
The series of stimuli started 10 min after the end of infusion. Two minutes between consecutive stimuli. Administration of 5 mg/kg reserpine subcutaneously 20—24 hours before the experiment. Both in control animals (not reserpinized, no infusion) and in reserpinized animals 0.5 mg/kg atropine was given intravenously in order to block the action of vasodilator nerves.
●———● Controls (mean of 4 experiments)
○———○ The sequence of stimulations was 3, 5 8, 10 and 15 impulses/sec. (Mean of 4 experiments.)
△— —△ *The sequence of stimuli was* 15 10, 8, 5 and 3 impulses/sec. (Mean of 4 experiments.)
The vertical bars represent ± *the standard error of the mean*

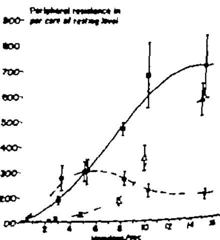


Fig. 3. *Peripheral resistance in percent of resting level during stimulation of the sympathetic chain after intra-arterial infusion of 100 µg adrenaline in reserpinized cats.*
●———● Controls. (Mean of 4 experiments.)
– – – – The sequence of stimuli was 3, 5, 8, 10 and 15 impulses/sec (Mean of 4 experiments.)
△— —△ The sequence of stimuli was 15, 10, 8, 5 and 3 impulses/sec (Mean of 4 experiments.)
For further details see legend to fig. 2.

stimuli were given in reserpinized animals. One frequency response curve shows the changes in peripheral resistance when the series of stimuli opened with the lowest frequency (3 imp/sec) followed by higher ones (5 8, 10 and 15 imp/sec). The other curve shows changes in peripheral resistance when the sequence of stimuli was reversed (15 10 8, 5 and 3/sec respectively). Although both curves differ from the normal one they do not, except in one instance differ significantly from each other. The one exception is that at the highest frequency (15/sec) the percentual change in peripheral resistance was greater when the series began with stimuli of that frequency than when the sequence was reversed.



Fig. 4 Cat 2.5 kg Reserpine 5 mg/kg. Urethane 600 mg/kg Vasomotor responses in skeletal muscle to stimulation of right sympathetic chain ($L - L_4$) before and after infusion of adrenaline and noradrenaline into the right femoral artery
1 Stimulation, 4 V 15 imp/sec.
2. 0.5 mg/kg atropine intravenously
3 = 1
4 100 µg adrenaline infused for 80 min.
5. Stimulation, 4 V 1 imp/sec. 10 min after infusion had ceased.
6. Stimulation, 4 V 3 imp/sec.
7 4 V 5 imp/sec.
8. 4 V 8 imp/sec.
9. 4 V 10 imp/sec.
10. 4 V 15 imp/sec.
11 = 6. 30 min after infusion had ceased.
12. = 7 13. = 8.
14. 100 µg noradrenaline infused for 85 min.
15. = 5. 10 min after infusion had ceased.
16. = 6, 17 = 7 18. = 8, 19 = 9 20. = 10.
21 = 6. 30 min after infusion had ceased.
22. = 7 23. = 8.
Note the rapid disappearance of vasomotor responses after adrenaline infusion, compared with the magnitude of the responses after noradrenaline infusion.

2. *Intra-arterial Infusion of Adrenaline*

Adrenaline (45—150 µg) was infused into 13 reserpinized cats. The infusion rate varied between 1.0—1.7 µg/min. Following a dose of 100 µg or more, sympathetic chain stimulation induced vasoconstrictor responses. Fig 3 shows the frequency-response curves after infusion of 100 µg of adrenaline. It is apparent that the frequency-response curves differ from the curves of normal cats, both in the stimulation series opening with a low and that opening with a high frequency For series opening with a low frequency the change in peripheral resistance was less at 10 and 15 imp/sec than at 3/sec or 5/sec. This phenomenon is illustrated in Fig 4 In this particular experiment both adrenaline and noradrenaline were infused i.a. Ten minutes after the infusion of adrenaline a series of sympathetic chain stimuli was initiated. The vasoconstrictor responses were greatest at 3 and 5 imp./sec after which they progressively diminished until, at 15/sec, they had almost disappeared. A series of stimuli initiated 30 min after the end of the infusion produced no change in blood flow (11 12 and 13) An identical dose of noradrenaline (100 µg) was then infused. Following noradrenaline there was no decrease of the vasoconstrictor responses at higher stimulation frequencies. Furthermore, vasoconstrictor responses still

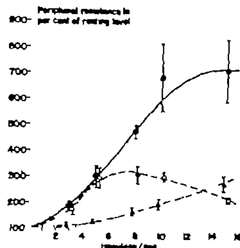


Fig. 5. *Peripheral resistance in percent of resting level during stimulation of the sympathetic chain after intra-arterial infusion of 100 μg dopamine.*
●———● Controls. (Mean of 4 experiments.)
○———○ The sequence of stimuli was 3, 5, 8, 10 and 15 impulses/sec. (Mean of 4 experiments.)
△— —△ The sequence of stimuli was 15, 10, 8, 5 and 3 impulses/sec. (Mean of 4 experiments.)
For further details see legend to fig. 2.

persisted 30 min after the infusion of noradrenaline had terminated. It would seem therefore, that infused adrenaline may be stored to a much lesser extent than infused noradrenaline at the nerve endings of the vasoconstrictor nerves in reserpinized cats.

3 *Intra-arterial Infusion of Dopamine*

In 18 reserpinized cats dopamine was administered i. a. the doses varying between 25—4 000 μg and the infusion rates between 2.5—6.7 μg/min. After low doses (25—400 μg) the vasoconstrictor responses to sympathetic chain stimulation were restored. With doses of about 400 μg or more, on the other hand, no vasoconstrictor responses were manifest at the lower stimulation frequencies (1, 3 and 5/sec). With still higher doses (2.5—4 mg) no vasoconstrictor responses whatsoever could be elicited in the infused hind limb. Studies on non-reserpinized cats showed that dopamine in comparatively high doses (1—4 mg i. a.) had a sympatholytic action of at least one hour's duration, apparently as a result of competition for the receptor sites between the infused material dopamine and the released transmitter substance. The failure of dopamine in high doses to restore the vasoconstrictor effects in the infused leg of a reserpinized cat may well be attributable to this sympatholytic action. Fig. 5 shows the stimulation frequency-response curves after infusion of 100 μg dopamine. It will be seen that even dopamine may to some extent restore the effects of the vasoconstrictor nerve activity in reserpinized cats. It was impossible, however, to elicit vasoconstrictor responses of the same nature as those obtained in normal cats.

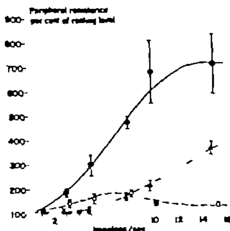


Fig. 6. *Peripheral resistance in percent of resting level during stimulation of the sympathetic chain after intra-arterial infusion of 5 mg dopa.*
●———● Controls. (Mean of 4 experiments.)
○— — —○ The sequence of stimuli was 3, 5, 8, 10 and 15 impulses/sec. Mean of 4 experiments.)
△— —▲ The sequence of stimuli was 15, 10, 8, 5 and 3 impulses/sec. (Mean of 4 experiments.)
For further details see legend to fig. 2.

4. *Intra-arterial Infusion of Dopa*

The vasoconstrictor effects in reserpinized cats were also reactivated by infusion of dopa, though only after high doses (2 mg or more. Infusion rate 0.08—0.25 mg/min). In 11 experiments dopa was infused in doses varying between 0.075—5.8 mg. Fig. 6 shows the stimulation frequency-response curves following infusion of 5 mg i. a. By comparison with the vasoconstrictor response obtained on stimulation of the sympathetic chain in non-reserpinized cats, the effects at all frequencies were slight.

Discussion

The biosynthesis of noradrenaline and adrenaline seems to proceed along the pathway suggested by BLASCHKO (1939). By introduction of a phenolic OH group into tyrosine, dopa is formed and in turn is decarboxylated to dopamine. Dopamine is then hydroxylated to noradrenaline and this in turn is methylated to adrenaline. We have studied the effects of dopa, dopamine, noradrenaline and adrenaline on the responses to stimulation of the vasoconstrictor nerves of skeletal muscle vessels in reserpinized cats. Each of the substances tested is in fact able to restore vasoconstrictor effects. There are, however important quantitative differences. From Fig. 1 it is evident that vasoconstrictor effects could be elicited at least one hour after the infusion of noradrenaline had terminated. The same was true of dopa and dopamine but not of adrenaline. These results are consistent with those of AXELROD (1960 a). He concluded that a greater fraction of administered noradrenaline than ad

ministered adrenaline appears to be bound to the tissues. Furthermore, EULER (1956, 1958) and SCHÜMANN (1958) on the basis of results obtained with homogenates of sympathetic nerves (bovine splenic nerves) showed that these nerves contain noradrenaline but not adrenaline granules. Moreover, FOLKOW and UVNÄS (1948) obtained evidence that the transmitter substance at the vasoconstrictor nerve endings consisted of noradrenaline rather than adrenaline.

It is interesting to note that sympathetic stimulation following administration of adrenaline induced vasoconstriction, never vasodilatation. As is well known adrenaline in low concentrations elicits dilatation in the skeletal muscle vessels of the cat but in high concentrations induces vasoconstriction. It is not likely that adrenaline was demethylated to noradrenaline which was then released by stimulation, since there is little evidence for the formation of noradrenaline from adrenaline (BACQ 1960, AXELROD 1960 b). The interpretation may be that the release of adrenaline by stimulation was presumably confined to certain restricted areas with a specialized type of receptor site whereas upon infusion of adrenaline there is a general distribution of the drug.

Infusion of dopamine also reactivated the adrenergic vasoconstrictor responses. The pressor action of dopamine is 50 to 100 times less than that of noradrenaline (BALZER and HOLTZ 1956). On the other hand, the threshold doses for restoration of the effects of the vasoconstrictor nerve impulses were about the same for noradrenaline and dopamine (25 μg). The major difference in the pressor effects of the two substances despite the near parity of the threshold doses suggests the conversion of infused dopamine to a more potent pressor amine, probably noradrenaline, and the subsequent release of the latter on stimulation of the vasoconstrictor nerves.

Dopa likewise had the capacity to reactivate the vasoconstrictor nerves. It is highly unlikely that dopa *per se*, which exhibits no direct vasoactive properties when administered intravenously or intra-arterially, would restore the effects of vasoconstrictor nerve activity. It is reasonable to suppose, therefore, *that the amino acid dopa underwent conversion to an amine.* One possibility is that dopa was decarboxylated to dopamine and the latter in turn hydroxylated to noradrenaline. In view of the quantitative relationships it is almost impossible, however, to conclude with assurance which substance was released during vasoconstrictor nerve stimulation after infusion of dopa. Even after large doses of dopa (5 mg i. a.) the vasoconstrictor responses did not exceed those observed following infusion of 100 μg dopamine. Assuming that formation of dopa to noradrenaline occurred in the hind leg, this may imply that at most 2 per cent of the infused dopa was converted to dopamine. The explanation of this small conversion has not yet been sought. In the sequence of reactions from tyrosine to noradrenaline the fastest reaction is presumably the decarboxylation of dopa to dopamine (for references see HOLTZ 1959). Thus the small conversion is probably not due to a slow rate of decarboxylation of dopa to dop-

amine. Moreover HOLTZ and WESTERMANN (1956) found a high dopadecarboxylase activity in splenic nerves, indicating that sympathetic nerves may form dopamine from dopa. GOODALL and KIRSHNER (1958) using radioactive dopa and tyrosine, studied the formation of adrenaline and noradrenaline in minced sympathetic nerves and ganglia. They reported that dopamine and noradrenaline were formed. These studies of HOLTZ and WESTERMANN and of GOODALL and KIRSHNER took form of *in vitro* experiments on homogenized splenic nerves. To some extent our results seem to support these findings. Thus it is probable that in the cat adrenergic vasoconstrictor nerve terminals or adjacent tissue form noradrenaline from dopamine whereas the evidence that the same tissue in reserpinized cats form dopamine from dopa is by no means conclusive.

BURN and RAND (1960) suggested that the restoration of the effects of sympathetic stimulation following an infusion of noradrenaline is due to the retention of noradrenaline in some form. Moreover they considered it likely that infused precursors undergo conversion in the direction of noradrenaline before they act. However MUSCHOLL (1960) has shown that in reserpinized rats (5 mg/kg) infusions of noradrenaline did not produce any increase of the noradrenaline concentration in the heart. On the other hand, in normal rats, the heart concentration of noradrenaline increased considerably after infusion of noradrenaline. These results of MUSCHOLL are in agreement with the assumption that reserpine blocks the active transport of catechol amines to storage granules (HUGHES and BRODIE 1959 BERTLER, ROSENGREN and ROSENGREN 1960) At first glance our experimental results and those of BURN and RAND appear to contradict this hypothesis. However experiments have been performed in which restoration of vasoconstrictor effects was tested 5 hours after the administration of reserpine. After such a short interval noradrenaline (50 μg i. a.) failed to restore the vasoconstrictor response of sympathetic chain stimulation (unpublished observations) Furthermore, in the experiments described in this paper 100 μg of noradrenaline could not restore the effect of vasoconstrictor nerve stimulation to a normal level (Fig 2) It is thus conceivable that the action of reserpine had partially subsided during the long interval between the administration of reserpine and the experiment (20–24 hours) In the experiments of MUSCHOLL reserpine was also administered 20 hours before beginning the experiment. However the duration of the action of reserpine might very well vary according to species and organ. Thus the experiments presented and those of BURN and RAND do not seem to contradict the hypothesis that reserpine blocks the active transport of catechol amines to storage granules.

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From the Department of Physiology University of Göteborg and the Institute of Physiology University of Lund, Sweden

Monosynaptically Evoked Inhibitory Post-Synaptic Potentials in Motoneurones

By

E. Eide, A. Lundberg and P Voorhoeve

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Abstract

Eide, E., A. Lundberg and P Voorhoeve. *Monosynaptically evoked inhibitory post-synaptic potentials in motoneurones.* Acta physiol. scand. 1961 53. 185—195 — Intracellular recording from motoneurones was made in cats with intact dorsal roots and in chronically de-afferented cats. Stimulation of the gray matter was performed with electrodes inserted into the spinal cord. Large excitatory and inhibitory post-synaptic potentials were evoked by these stimuli in alpha motoneurones after a latency of about 0.5 msec. It is concluded that excitatory and inhibitory interneurones were activated and that the synaptic delay at inhibitory and excitatory synapses is the same.

The first measurement of synaptic delays in the central nervous system was made by Lorente de Nó (1935) who found a minimal delay of 0.5 msec for transmission of excitation to motoneurones. Later investigations with recording of the onset of the monosynaptic excitatory postsynaptic potential (EPSP) relative to the presynaptic volley have given values of 0.3—0.35 msec (Brock, Coombs and Eccles 1952)

Estimates of the synaptic delay at inhibitory synapses in the central nervous system rest on indirect evidence. The relevant investigations have largely been made on the Ia inhibitory pathway the start being Lloyd (1946) description of direct inhibition, i.e. the inhibition of monosynaptic reflexes caused by I impulses from the antagonist muscle. Lloyd found that this inhibition was discernible when the inhibitory and excitatory volleys arrived approximately simultaneously at the spinal cord and concluded that the inhibitory action was exerted monosynaptically the inherent assumption being that the

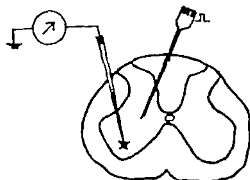


Fig. 1. Drawing of the arrangement of stimulating and recording electrodes.

synaptic delay at inhibitory and excitatory synapses is of the same order. With intracellular recording of the inhibitory post-synaptic potential (IPSP) the central latency for the Ia inhibitory synaptic action could be measured accurately and was found to be 1.3 msec or about 0.8 msec longer than for the monosynaptic Ia EPSP (BROCK *et al.* 1952).

In 1956 ECCLES, FATT and LANDGREN postulated that an interneurone is interpolated in the Ia inhibitory pathway and that the delay at inhibitory synapses would be of the same order as at excitatory or about 0.3 msec. Investigations on other inhibitory pathways from somatic afferents to motoneurones revealed central latencies for the IPSPs that were so long in relation to the assumed synaptic delay of 0.3 msec that one or several synaptic relays were postulated (ECCLES *et al.* 1956, ECCLES, ECCLES and LUNDBERG 1957 b, CURTIS, KRNJEVIC and MILEDI 1958, ECCLES and LUNDBERG 1959, FRANK and SPRAGUE 1959). The same holds true for the recurrent Renshaw inhibition from motor axon collaterals (ECCLES, FATT and KOKETSU 1954) and for the inhibitory action exerted by impulses in descending spinal pathways (PRESTON and WHITLOCK 1960). Similar findings have also been made on the cells of the dorsal and ventral spino-cerebellar tracts (CURTIS, ECCLES and LUNDBERG 1958, ECCLES, HUBBARD and OSCARSSON 1961, ECCLES, OSCARSSON and WILLIS, 1961). Pending confirmation of the basic assumption these findings add support to the generalizing suggestion (ECCLES *et al.* 1956, ECCLES 1957) that every inhibitory pathway in the central nervous system has a final stage short-axon inhibitory interneurone.

LLOYD and WILSON (1959) have recently claimed that for the Ia pathway the IPSP is not the primary agent of the inhibition but this claim has been effectively refuted by ARAKI, ECCLES and ITO (1960).

In order to obtain further evidence on the synaptic delay at inhibitory synapses it would be desirable to record monosynaptically evoked IPSPs. In the present experiments a stimulus has been applied through electrodes inserted into the gray matter and the latency of IPSPs and EPSPs evoked in motoneurones has been found to be about 0.5 msec. A preliminary report has been given (EIDE, LUNDBERG and VOORHOEVE 1960).

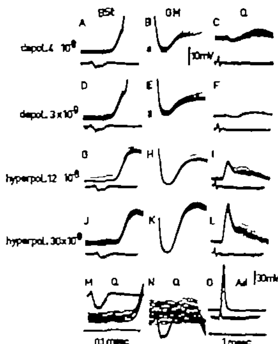


Fig 2. Intracellular recording with KCl-electrode from posterior biceps-semitendinosus (BSt) motoneurone, internal positivity (depolarization) being signalled upwards. Lower traces (except in M) were recorded from the dorsal root entry zone. A, D G and J in the left column were obtained on stimulation at constant strength of the BSt nerve. In B, E, H and K the gray matter (GM) was stimulated at constant strength through electrodes inserted approximately as shown in Fig 1 The quadriceps nerv was stimulated at constant strength in C, F I and L, which were recorded at the slow sweep speed, and in M and N at faster sweep speed. The S 1 ventral root was stimulated in O. Curren was passed through the recording electrode and for each horizontal row the current flow is indicated in amperes.

Methods

The experiments were made on 5 cats under light anaesthesia from pentobarbitone sodium. In tw experiments the dorsal roots were intact and in the remaining three the animals were de-afferented by removal of the spinal ganglia L6—S1 1—3 weeks before the experiment. The spinal cord was transected in L1 and the ipsilateral latero-ventral funicles were dissected for stimulation. I one experiment the dorsal part of the lateral funicle and the ventral quadrant were dissected for separate stimulation. Intracellular recording was made in the L7 and S1 segment as described by Brock *et al.* (1952) The recording micro-electrode was inserted from the lateral aspect of the cord. The steel electrodes used for stimulation were insulated, except for the tip, and inserted through the dorsal column as shown in Fig 1 The stimulating electrodes were inserted to depth of 2 5—3 mm. The distance between the tips of the stimulating electrodes was about 0.5 mm, the ventral electrod being cathode Square wave pulses with duration of 0.1 msec were delivered via radio frequency isolation unit.

All records consist of many superimposed traces.

Results

A comparison f synaptic actions evoked by impulses in primary afferents and by stimulation of the gray matter

The intracellular records in Fig 2 are from a posterior biceps-semitendinosus (BSt) motoneurone and show the synaptic potentials evoked on stimulation of the BSt nerve (A, D G J) of the gray matter (GM) (B E, H, K) through steel electrodes inserted as shown in Fig 1 and of the quadriceps nerve (C,

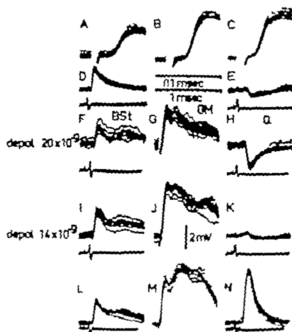
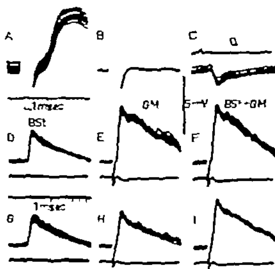


Fig 3. As in Fig 2, intracellular recording with KCl-electrode. Stimulation of the gray matter at increasing strength in A—C and at constant strength in G, J and M. The BSt nerve was stimulated in D, F, I and L and the quadriceps nerve in E, H, K and N. The lower horizontal rows of records were obtained at the indicated current flow through the recording electrode. A—C were obtained at the fast, all the other records at the slow sweep speed.

F, I, L, M, N). The recording micro-electrode was filled with 3 M KCl-solution and in order to analyse inhibitory and excitatory components of the post-synaptic potentials (PSPs) evoked by GM stimulation, current was passed through the recording micro-electrode to change the membrane potential (COOMBS, ECCLES and FATT 1955). At a depolarizing current of 4×10^{-9} A, the Ia IPSP from the quadriceps (C) was in the hyperpolarizing direction but when the current was decreased to 3×10^{-9} A the Ia impulses from quadriceps (F) hardly evoked any change of the membrane potential (cf. COOMBS et al. 1955). Hence the depolarizing effect in E shows the size of the EPSP evoked by the GM stimulus. The latency from the shock artefact to the onset of the EPSP in F is 0.50 msec as compared with the central latency of 0.55 from the Ia EPSP evoked from the BSt nerve in D (measured from the incoming volley, lower trace, to the onset of the EPSP). When the membrane was hyperpolarized so as to reverse the Ia IPSP from quadriceps (I and L) the depolarizing effect of GM stimulation increases in H and K. This increase in depolarization from E must be due to the superposition of a reversed IPSP. This is proved by the fact that the Ia EPSP from the BSt nerve does not change proportionally (I and J). A comparison of record E and K reveals that the reversed IPSP in K and the EPSP in E have a similar time-course and that the latency of the IPSP is of the same order as of the EPSP. The latency of the depolarization in K is 0.46 msec as compared with 0.50 for the EPSP in E. This difference is however hardly significant since the onset is easier

Fig 4. As in Fig. 2, intracellular recording with KCl electrode. Stimulation of the gray matter in A, B, E and H. B shows the extracellular field after a slight withdrawal of the recording electrode from the cell. The maximal homonymous EPSP from BSt is shown in D and G. F and I were obtained on stimulation of the BSt nerve and of the gray matter at the same strength that was used to evoke the responses in E and H respectively. In record C the quadriceps nerve was stimulated. This record, obtained at the end of the series, shows that the IPSP was still in the hyperpolarizing direction. A and B were taken at the fast and all the other records at the slow sweep speed.



to measure accurately in K than in E. For comparison the onset of the Ia IPSP from quadriceps is shown at fast sweep speed in M where it is reversed and in N during passage of a depolarizing current (6×10^{-9} A). The central latency is 1.5 msec as found by BROCK et al. (19 ?)

In other BSt cells GM stimulation evoked only a short-latency EPSP with no evidence of an early IPSP. This is illustrated in Fig 5. Records A—C, at fast sweep speed, show synaptic potentials obtained at increasing strength of GM stimulation. Record E, taken afterwards illustrates that the Ia IPSP from quadriceps had not reversed, hence the synaptic potentials in A—C are EPSPs. In F—N the synaptic potentials, evoked from the BSt nerve (F I L) from GM stimulation (G J M) and from the quadriceps nerve (H, K, N) are shown during passage of current through the recording micro-electrode. When during hyperpolarization the Ia IPSP reverses (N) this occurs without increase of the short-latency PSP in M, hence there is no evidence that the GM volley in this case evoked any short-latency IPSP although record M gives evidence of a polysynaptic IPSP

It would be expected that GM stimulation activates Ia afferents and this is proved by the records in Fig 4. The Ia EPSP from the BSt nerve is shown in D and the EPSP evoked by strong GM stimulation in E. With combined stimulation of the BSt nerve and the gray matter in F there was hardly any increase from E, which indicates a considerable but not complete occlusion. On the other hand with the weaker GM stimulus in H there is only moderate occlusion. The size of the EPSP in I is 77 of the sum of the EPSPs in G and H. Hence in Fig 4 many of the Ia afferents were activated in the high threshold range of GM stimulation. BSt motoneurones receive Ia EPSPs from gracilis (ECCLES, ECCLES and LUNDBERG 195 a) but this action is

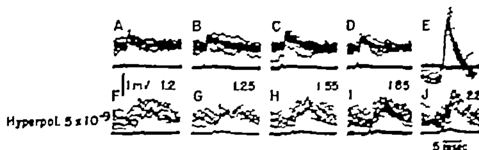


Fig. 5. Intracellular recording with a KCl-electrode from a gastrocnemius-soleus motoneurone. Record E shows the maximal homonymous EPSP from the gastrocnemius-soleus nerve. The ipsilateral dissected latero-ventral funicles were stimulated in A—D and F—J. Corresponding records in A—D and F—J were obtained at the same strength which is indicated, expressed as multiples of the threshold strength. Hyperpolarizing current (5×10^{-9} A) was passed through the micro-electrode in F—J and in E. The distance from the stimulating cathode in L1 to the site of micro-electrode recording in S1 was 50 mm. All the records were obtained at the same amplification.

small and cannot account for the large difference in size between the EPSPs in D and E. There are descending pathways with monosynaptic excitatory action (Fig. 5) but as will be discussed in the next section there is evidence that they were not activated by a stimulus applied as shown in Fig. 1 (see below). It is therefore assumed that excitatory interneurones were stimulated.

Intracellular recording was also used to investigate the effect of volleys descending from the upper lumbar region. Monosynaptic EPSPs were evoked from the ipsilateral ventral quadrants (presumably from the vestibulo-spinal tract) but no short latency IPSPs were found on stimulation of the ipsilateral or the contralateral latero-ventral funicles. The briefest segmental latency found was 1.3 msec as is illustrated in Fig. 5. A KCl-electrode was used for recording and in A—D only the monosynaptic EPSP appears. Hyperpolarizing current was passed through the electrode in F—J and a second depolarizing wave appears, which must be a reversed IPSP. The onset of this reversed IPSP which is evoked by activity in low threshold fibres occurs after a latency that is 0.9 msec longer than for the monosynaptic EPSP. Hence it can be concluded that this inhibitory connection is disynaptic. It can further be postulated that the IPSP evoked by stimulation of the gray matter in Fig. 2 is due to stimulation of interneurones and not of long descending pathways with monosynaptic inhibitory action on motoneurones. It is possible that the inhibitory interneurones activated to evoke the IPSP in Fig. 2 belonged to the Ia inhibitory pathway but no attempt was made to prove this. There are other ipsilateral inhibitory pathways from various hind-limb afferents to flexor motoneurones (HAGBARTH 1952, ECCLES and LUNDBERG 1959, HOLMQVIST and LUNDBERG 1959). In addition there must be interneurones mediating crossed inhibitory actions and presumably also interneurones mediating inhibition from descending pathways.

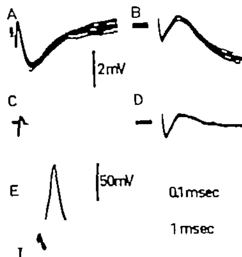


Fig. 6. The experiment was made on chronically de-afferented cat with intact ventral roots. Recording was made with K_2SO_4 electrode. The gray matter was stimulated in A—D at a constant strength. A and B were recorded intracellularly and the corresponding records in C and D extracellularly after slight withdrawal of the recording electrode. The antidromic response in E was evoked from the gastrocnemius-soleus nerve. A, C, E were obtained at the slow and B, D at the fast sweep speed.

Synaptic actions evoked by stimulation of the gray matter in chronically de-afferented animals

In order to facilitate the analysis experiments were also made on chronically de afferented animals after degeneration of the primary afferents, one to three weeks after removal of the ipsilateral spinal ganglia L6—S1. Recordings were made with electrodes filled either with 0.6 M K_2SO_4- or with 3 M KCl-solution. With sulphate electrodes the IPSPs do not reverse. KCl electrodes, on the other hand, have the advantage of lower resistance and also permit analysis of the admixture of composite PSPs by the passage of current.

The records in Fig. 6 were obtained from a gastrocnemius-soleus motoneurone and the slow conduction velocity of its axon (record E) indicates that it belonged to soleus although this was not verified by measurement of its after potential (*cf* ECCLES, ECCLES and LUNDBERG 1958). The records show the IPSP evoked by GM stimulation at two sweep speeds in A and B and the corresponding records below are the extracellular fields in response to an identical GM stimulus after withdrawal of the recording electrode. The latency of the IPSP in B is 0.53 msec.

The records in Fig. 7 are also from a gastrocnemius-soleus motoneurone. The recording electrode was filled with 3 M KCl. In order to prevent the IPSPs from reversing a depolarizing current of 10×10^{-9} A was passed through the electrode in A—D. The IPSP evoked by GM stimulation is shown in A at slow and in B at fast sweep speed and should be compared with the extra cellular fields in M and N. Records C—D show the Renshaw IPSPs (*cf* ECCLES *et al.* 1954) evoked from the indicated nerves at this level of depolarization. In E—H obtained without current flow these IPSPs reversed and actually excited the motoneurone. The series I—L in Fig. 7 was obtained

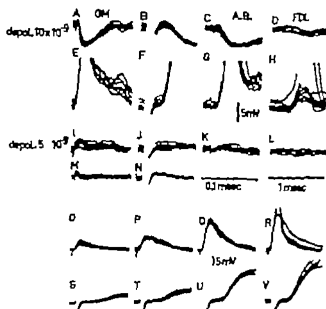
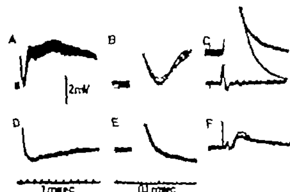


Fig. 7. Recording with a KCl-electrode in chronically de-afferented cat with intact central roots. A—L and O—V are intracellular records from gastrocnemius-soleus motoneurone; M and N were obtained extracellularly after slight withdrawal of the electrode. The grey matter was stimulated at constant strength in A, B, E, F, I, J, M and N and at increasing strength in the two lower rows (O—V) where corresponding records were evoked at the same strength but recorded at different sweep speeds. The nerves to anterior biceps (A. B.) and to flexor digitorum longus (FDL) were stimulated in C, G, K and D, H, L respectively. A depolarizing current of the strength indicated was passed through the recording electrode in A—D and I—L. Reversal of the IPSPs in E—H and O—V occurred without any passage of hyperpolarizing current. B, F, J, N and S—V were obtained at the fast, all other records at the slow sweep speed. Observe that records O—V were taken at a lower amplification.

with a depolarizing current of 5×10^{-9} A chosen to maintain the membrane potential at equilibrium level for the Renshaw IPSPs as is illustrated in records K and L. Under these conditions GM stimulation is also ineffective in evoking a synaptic potential and it can therefore be concluded that few or no excitatory interneurones with effects on this cell were activated by the GM stimulus. This conclusion is based on the *assumption* that the Renshaw IPSP is pure and does not conceal an excitatory effect. Previous investigations have not given evidence of an excitatory admixture in nembutalized spinal cats (ECCLES *et al.* 1954) although this occurs in unanaesthetized preparations (WILSON 1959).

In the de-afferented cats successful measurements were made on 14 flexor and 21 extensor motoneurones. The mean latency for EPSPs was 0.54 and for IPSPs 0.52 msec. The small difference is probably entirely explained by the fact that the onset of the IPSP could be more accurately measured since in many of the cells current was passed to reverse the IPSP and with this procedure it was easier to measure the onset. In the majority of motoneurones to extensors the inhibitory effects dominated, whereas in flexor motoneurones excitatory effects were prevalent. The majority of interneurones stimulated are probably concerned with transmission from hindlimb afferents and this distribution may reflect the flexor reflex pattern. However in a few extensor motoneurones excitation dominated as shown in the gastroc-

Fig. 8. Recording with K_2SO_4 electrode in chronically de-afferented cat. A—C and F are intracellular records from gastrocnemius-soleus motoneurone. D and E are the extracellular fields obtained after slight withdrawal of the recording electrode. The grey matter was stimulated at constant strength in A, B, D and E. A and D were recorded at slow, B and E at fast sweep speed. The gastrocnemius-soleus nerve was stimulated in C and the ipsilateral latero-ventral funicles dissected in L2 are stimulated in F



nemius-soleus motoneurone of Fig. 8 there is a short latency monosynaptic EPSP (latency in B 0.58 msec) as well as a later polysynaptic EPSP which can be observed at the slow sweep speed in A. Record F shows the maximal monosynaptic EPSP evoked by a descending volley in the ipsilateral latero-ventral funicles (probably in the vestibulo-spinal tract). It is assumed that the EPSPs evoked by GM stimulation are due to activation of interneurones and not of these descending fibres. Our reason for this assumption is that in a number of cells in which GM stimuli failed to evoke EPSPs at a strength slightly submaximal for direct excitation of the motoneurone relatively large monosynaptic EPSPs were nevertheless evoked by the descending volley. A more strict proof could have been provided by experiments on chronic spinal and de-afferented animals, but in this investigation we were concerned mainly with the inhibitory effects.

Discussion

The "inhibitory interneurone" hypothesis (ECCLES *et al.* 1956) was based on experiments with the Ia inhibitory pathway to motoneurones and until now the evidence that an interneurone is interpolated in this path was threefold.

1) Ia volleys from the quadriceps nerve evoke spike potentials in the posterior biceps-semitendinosus nucleus after a central latency of about one msec, thus preceding the onset of the IPSP by 0.3 msec. These spike potentials presumably represent activity in preterminal fibres of the Ia inhibitory interneurones to BSt cells (ECCLES *et al.* 1956).

2) The connection of group Ia fibres in the lumbar cord is entirely in agreement with a postulated relay in the intermediate nucleus of CAJAL and there is anatomical and physiological evidence that the axons of these cells terminate on motoneurones or in motor nuclei (SZENTÁGOTHAI 1951, ECCLES, FATT, LANDGREN and WINSBURY 1954, ECCLES *et al.* 1956, CURTIS 1959, *cf.* ECCLES 1957, 1961).

3) Spatial facilitation has been demonstrated in the Ia inhibitory pathway (ECCLES and LUNDBERG 1958). This was shown in different ways, the most spectacular being the experiments in which stimulation of any one of 5 branches from the quadriceps nerve failed to evoke a Ia IPSP in BSt motoneurones, whereas a large IPSP could be recorded on simultaneous stimulation of these branches.

Altogether these findings constitute strong circumstantial evidence in favour of the inhibitory interneurone hypothesis but in view of the paramount importance of this hypothesis it was desirable to test it to the utmost. The only possible alternative would be to assume that the delay at inhibitory synapses is longer than at excitatory. The present experiments have excluded this possibility. The onset of the IPSP evoked by a stimulus applied to the gray matter occurred after a mean latency of 0.52 msec and a minimal latency of 0.46 msec. The corresponding values for the EPSPs evoked by these stimuli were 0.54 and 0.50 respectively. It has already been pointed out that the difference in latency between the EPSP and IPSP is *not significant*. *It is concluded* that the synaptic delay at excitatory and inhibitory synapses is the same.

It was to be expected that the latency for PSPs evoked by electrical stimulation should exceed the one derived by BROOKS and ECCLES (1947), BROCK *et al.* (1952) and ECCLES *et al.* (1956). The utilization time for the stimulus and the conduction time to the terminals probably accounts for most of the difference. On the other hand measurements of the interval between the presynaptic spike and the onset of the PSPs may give values for the synaptic delay on the low side, because the onset of the PSP depends on the fastest fibres, whereas the mean velocity is measured in the presynaptic recording.

There was no indication that fibres in the upper lumbar region have monosynaptic inhibitory connections with motoneurones in L7 and S1 but a disynaptic inhibitory connection was found (Fig. 5) (*cf.* PRESTON and WHITLOCK 1960). The monosynaptic IPSPs evoked on stimulation of the gray substance presumably are due to activation of interneurones and the same is postulated for the EPSPs evoked in the chronically de-afferented cats. The stimulation technique could be used to locate excitatory and inhibitory interneurones which terminate on motoneurones.

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From the Department of Physiology Faculty of Medicine, Karolinska Institutet, Stockholm, Sweden

Uptake of Catecholamines by Adrenergic Nerve Granules

By

U S von Euler and F Lishajko

Nerve granules were prepared from bovine splenic nerves (Euler and Lishajko 1961) and incubated at room temperature and pH 7.0 in isotonic potassium phosphate to which catecholamines had been added in different concentrations. In the presence of 10 μg noradrenaline (NA) per ml in the incubation fluid no spontaneous loss of the granule-bound NA was noted in 60 min at 20 C as against a loss of 67 per cent when the NA concentration was 1 μg per ml or less in the incubation fluid No loss was observed after incubation even for 120 min in NA 20 μg/ml with 5 μg/ml NA the loss was 50 per cent during the same time.

After previous incubation of the granules for 120 min causing a depletion of about 80 per cent, and subsequent incubation for 60 min with NA after sedimentation and resuspension of the granules, an uptake of NA was observed. This was small but measurable with 5 μg/ml NA in the incubation fluid and increased with raising concentrations of NA. With 20 μg/ml NA in the incubation fluid the granules were repleted up to 95 per cent of the original amount whereas the controls had lost over 80 per cent of their NA. Reserpine 10 μg/ml did not prevent the uptake of NA by depleted granules. Adrenaline was taken up by nerve granules in similar amounts as NA.

The results show that isolated nerve granules can take up catecholamines which has previously been shown to occur for dopamine in adrenal medullary granules (Bertler et al 1961) Assuming similar conditions in vivo as in vitro, it might be hypothesized that while NA is retained in the storage granules when the concentration in the axoplasm is about 10 μg/ml, as actually found in vivo, it may be released from the granules at lower extragranular concentrations following NA flux through the axon membrane during nerve stimulation.

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From the Laboratory of Biophysics, The Rockefeller Institute, New York, U. S. A.

A Self Recording Electronic Osmometer for Quick, Direct Measurement of Colloid Osmotic Pressure in Small Samples[1]

By

A. Tybjaerg Hansen[2]

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Abstract

Hansen, A. T. *A self-recording electronic osmometer for quick, direct measurement of colloid osmotic pressure in small samples.* Acta physiol. scand. 1961. 53. 197–213. — A self-recording electronic osmometer for clinico-physiological use is described. It is further development of an osmometer described earlier by the author. It is based on the passive counter-pressure principle and as such representing return to the principle that was first applied in osmotic pressure measurements. The general principles for the measuring of osmotic pressure and the special requirements to be fulfilled if quick performance is to be obtained are discussed. The importance of the diffusion phenomena in this context has been emphasized and experimentally elucidated. The sample of plasma or serum which is required, is from 0.02 to 0.1 ml. Ten to 15 measurements can be performed per hour. The confidence limits for one measurement are ± 0.5 mm Hg. t the 5 level. Commercially available cellulose membranes are used. N stopcocks are employed. Stainless steel is used for all parts in contact with membrane and sample. The possible usefulness of the osmometer for investigation of the reflection coefficient of permeating particles and for testing membranes is pointed out. Also the possible use in investigations of active trans port problems in connection with organic membranes is hinted t. Finally it is mentioned that the red blood cells under certain circumstances will influence the osmotic pressure

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Sophus Fricke Fellow of The Royal Danish Academy of Sciences and Letters in The Rockefeller Institute.

Present address Rigshospitalet, Copenhagen.

In spite of the prominent role the colloid osmotic pressure (COP) is supposed to play in the circulation of the blood (STARLING 1896) necessary data for an evaluation of its significance in health and disease are still insufficient. This is to a great extent now as before the result of the lack of entirely satisfactory methods for clinico-physiological studies of the COP.

The *indirect methods* can hardly be considered in exact measurements. Quantitative chemical analysis and ultrafiltration yield results which can only be translated into COP with a wide margin of uncertainty in the individual cases. Freezing point determination and other methods based on colligative properties will fail because of the very low osmolar concentration of the proteins, particularly in the presence of crystalloids, as in the body fluids.

Direct osmometry is the method of choice, but the long time involved in the single measurement, the demand for large samples and the cumbersome procedures in general have restricted the use of the technique in clinical investigation.

By the introduction of electronic pressure transducers into the direct osmometry (TYBJAERG HANSEN 1950, 1952, MEEHAN *et al.* 1950, PAPPENHEIMER and LIN 1953, ROWE 1954) the possibility of reducing the measuring time as well as the size of the sample was significantly improved. In the following it will be shown that this principle, if thoroughly exploited, permits the design of an osmometer that is rapid, simple and dependable enough for routine use in clinical physiology.

Common principles and problems in direct osmometry with a special view to clinico-physiological demands

The direct measurement of the osmotic pressure of the solution requires a so-called semipermeable membrane. That is a membrane permeable to the solvent but impermeable to the solute. When solvent and solution are placed on either side of the membrane contiguity is obtained through the interstices and osmotic flow begins from the solvent into the solution. This flow will continue as long as there is fluid left in the solvent compartment, at least theoretically. The mechanism by which this flow is generated is not fully understood (MAURO 1957). However it is influenced by hydrostatic pressure as is any other hydraulic flow and can therefore be slowed, stopped or reversed if a suitable pressure difference is established between the two compartments. The pressure difference, when no net flow takes place in either direction, is per definition the osmotic pressure (π). It is proportional to the osmolar concentration (C) and the absolute temperature (T) with the gas constant (R) as proportionality factor according to van t Hoff' equation ($\pi = RTC$). It should be noted that π is independent of the species of the solute molecule.

The zero flow criterion is the basis for all direct osmometry, and this implies that detection of volume flow across the semipermeable membrane is an essential part of the measurement. The volume displacement can be observed directly or

be indicated by its effect on the pressure in one or more rarely both of the compartments.

In the so-called static methods (WAGNER 1949) here to be denoted *passive* the balancing pressure difference is caused by the osmotic flow itself. The equilibrating time is proportional to the relation between the volume displacement and the pressure change it causes.

In the dynamic methods (WAGNER 1949) here to be denoted *active* the volume displacement across the membrane is only significant as a means to guide the adjustment of an "externally" applied pressure so as to bring about zero flow conditions. For the sake of convenience one of the compartments is as a rule kept at atmospheric pressure, just as the case is in the passive methods.

The active method was introduced (TAMMANN 1892) in order to reduce the extremely long measuring time required by the passive methods. The reason for the slowness of the said methods is the very high flow resistance characteristic of semipermeable membranes. As the necessary liquid displacement across the membrane in principle could approach zero in the active methods the problems of the protracted measuring time seems to be satisfactorily resolved. However the period of fine adjustment in the active method is not much less time consuming than the last part of the approach to equilibrium pressure in a comparable passive method, *i. e.* with the same sensitivity for detecting volume displacement.

The basic consideration in attempts to reduce the measuring time is, therefore, to increase the sensitivity with which the volume displacement across the membrane can be detected, *i. e.* to increase the modulus of volume elasticity (TYBJÆRG HANSEN 1949).

Electronic methods

It is first of all in this context that the opportunities of electronic methods must be viewed, although they offer other technical advantages, e.g. convenient possibilities of recording. When suitable transducers are employed a very high modulus of volume elasticity can be obtained. The extent to which this favourable property can be exploited depends on the proper design of the rest of the osmometer.

The considerations of *ultra-small volume displacements* do not pose any basically new problems but some of the old ones assume new relative dimensions.

This is true with respect to the *support of the semi-permeable membrane*. A "rigid" support in its practical sense is relative and means that the degree to which the semipermeable membrane yields to pressure is small compared to that of the pressure-sensitive parts, in both cases expressed as volume. The support of the membrane is, therefore, particularly critical when, as here, ultra-small volume displacements are in question. The deleterious effect of an unduly yielding semipermeable membrane cannot be compensated for by any counter-pressure procedures, a fact which has not always been fully realized.

A strictly air-free *filling* of the compartment in which the indicating pressure is to be detected is, of course, necessary in order to sustain the high modulus of volume elasticity of the transducer.

For the same reason the *amount of liquid* held in the pressure chamber of the osmometer should not be too large, as the compressibility of water is not negligible compared to the compliance of the pressure-sensitive part itself (Tybjærg Hansen 1949).

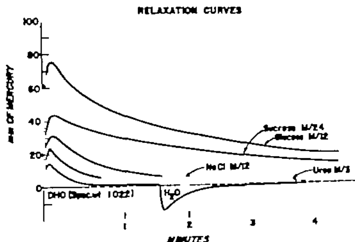


Fig. 1 The curves show the effect of ultimately penetrating substances. The osmotic pressure is related to the osmolar concentration difference at any moment and to the reflection coefficient for the solute in question. The DHO concentration is about 10 M (18 °/₀) The initial reference fluid is in all instances distilled water except in the case denoted H_2O where it is DHO of approximately 10 M concentration. Urea has a remarkably low reflection coefficient.

Whereas for the most part the ultra-small volume displacement leads to exacting demands on the design, it makes it easier in one respect in that it offers the opportunity of reverting to the less complicated passive method.

Even though the preservation of the high volume elasticity coefficient is the primary consideration, attention must be paid to other factors influencing the measuring time, most of them having to do with the properties of the *membrane* itself.

The *water permeability* of the membrane should be as high as possible. Everything else being equal this will mean a membrane with the most numerous and largest possible "pores" i. e. just small enough to withhold the protein molecules. This property is by and large determined by the material of and the manner in which the membrane is mad but in addition the *thickness* and the *area* of the membrane are, of course, determinative.

A reduction of the thickness will facilitate the volume flow So will an enlargement of the area but in both cases it will become harder to secure a rigidly supported, unyielding membrane. Furthermore an enlargement of the area runs counter to the desire of using a small sample, which is important in a number of situations met with in clinical physiology

Although the reasoning on the whole leads to the recommendation of relatively thin membrane of small area, the actual dimensions must be chosen as a compromise on the basis of experience.

A matter of particular interest is the *influence of diffusion*, which has been especially clearly brought out during the work with the present apparatus. Generally speaking swift diffusion runs parallel to high permeability of a membrane. The thickness of the membrane plays the same part in connection with diffusion as it does with solvent flow The area, however does not influence the time it takes to reach diffusion equilibrium as long as the same relative proportions exist between the area and the volumes in which diffusion takes place.

In order to obtain a quick establishment of the diffusion equilibrium it is favourable to reduce the amount of fluid on both sides of the membrane, preferably to thin layers.

However in order to avoid changes in the salt concentration of the sample it must be large in comparison with the volume of the reference fluid, or rather with that of the effective reference volume. This is defined as that part of the fluid within the solvent compartment in which diffusion equilibrium with the sample has to be obtained and maintained.

The least possible effective reference volume will be that of the membrane or more precisely the volume of solvent in the membrane. It is possible to approach this very closely as will be shown.

The reason why diffusion equilibrium is so important to establish in connection with measurement of COP is that the penetrating molecules and ions exert their osmotic effect according to their concentration at any moment and to their reflexion coefficient (STAVERMANN 1952). Although their effect is equal only to a small fraction of their full osmotic effect as estimated from their osmolar concentration this effect is not at all negligible compared with the COP. Furthermore, and this ought to be particularly stressed, the establishment of diffusion equilibrium cannot be hastened by use of any of the active methods, as diffusion is not much influenced by pressure (MAURO 1957).

In *Fig. 1* is shown how permeating molecules even as small as DHO make themselves osmotically manifest as recorded by the osmometer here to be described.

As long as the diffusion equilibrium is not established the COP measurement cannot be completed. The diffusion conditions are therefore a potentially limiting factor in connection with attempts to reduce the measuring time. Besides reduction of the effective reference volume, as mentioned above, stirring of the sample and selection of the reference fluid before the measurement may be used as a means to enhance the establishment of diffusion equilibrium.

Choice of sample compartment in relation to pressures

The solvent flow is dependent on the pressure difference and not on the absolute pressures in the two compartments. The pressure difference indicating the osmotic pressure may therefore, be arrived at in several different ways.

From a practical point of view it is highly recommendable to maintain the sample under atmospheric pressure and secure the proper pressure difference by means of a negative (subatmospheric) pressure in the compartment containing the reference fluid. This makes it simple to change a sample, and it prevents contamination of the manometric chamber with protein.

Design and description of the osmometer

The principles here expounded were rather successfully applied in the first version of the osmometer but it has been possible to exploit the principle more fully in the new one here to be described.

The transducer itself (TYBJÆRG HANSEN 1949) is only slightly modified. The volume of the pressure chamber is reduced to 0.5 ml. The modulus of volume elasticity is slightly higher than the 4 × 10⁹ dynes × cm.⁻² of the old one.

The electronic part of the apparatus represents an improvement in performance but there are no principal changes. Only the oscillator and converter but not the amplifier are used. The output from the converter is fed into a

Universal Indicator DISA ELEKTRONIK, Copenhagen, Denmark.

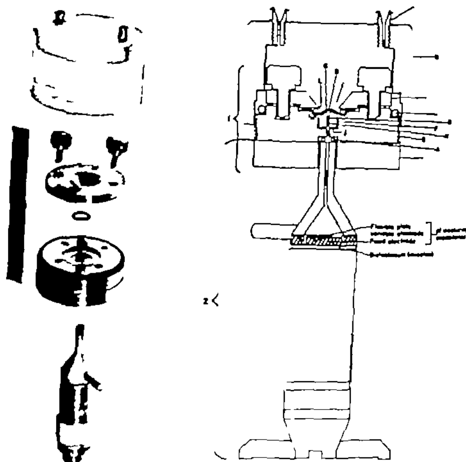


Fig. 2. Axial section of the osmometer and exploded view. Explanation in text.

standard Varian Recorder. The modifications of the transducer and the electronic equipment will be described elsewhere in another context.

The first version of the osmometer was designed as an active method in accordance with the afore mentioned principles. However as it became evident that it would be possible to achieve equilibrium within a very short time even with a passive method all attention was given to develop the osmometer accordingly so as to avoid the troublesome counter-pressure procedures of the active methods. This decision was further encouraged by the circumstance that the reduction of equilibrating time had reached a degree where active methods had no particular advantages because of the relatively greater influence of the diffusion conditions such as stated above.

Type G-10, full scale deflection 0 to 10 mV in 2.5 seconds. Paper speed 4 inches/hour and 1 inch/minute.

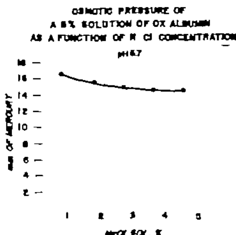


Fig 3. The effect on the osmotic pressure of varying salt concentrations is result of the adaptation of the Donnan equilibrium. It appears from the curve that the effect is only slight.

In order to make full use of the high modulus of volume elasticity the stopcock that was present in the first version was abandoned in order to secure absolute tightness. The result was a noticeabl simplification of the osmometer which now consists of two principal parts as shown in *Fig. 2.* and *3*

The pressure-sensitive transducer (2)

The clamping unit. (1)

The two parts are assembled by a threaded plug and socket arrangement. The leak proof sealing is accomplished by an O-ring (A) This arrangement will not only make sure that the system be tight without impairing the modulus of volume elasticity but also that the inner volume and consequently the pressure can be easily changed just by turning the clamping unit slightly with regard to the transducer

The *clamping unit* (1) is made of stainless steel. The decisive feature is the arrangement for the support of the semipermeable membrane. In contrast with that of the first version the supporting cylinder (B) has a completely smooth surface (C) without grooves of any kind. The membrane (D) rests directly on this surface. The convexity of the surface is increased compared with the first model. The supporting cylinder (B) fits tightly into its socket in the body of the clamping unit (E) leaving a thin, concentric, cylindrical space (F) as main connection to the manometric chamber (G) In the centre of the cylinder is drilled a tiny channel (H) that just permits the insertion of a 27 gauge needle. This arrangement facilitates flushing th space (J) just beneath the supporting cylinder and the concentric space mentioned above. The surface f the supporting cylinder is flush with the immediately surrounding, slightly recessed surface (K) of the body of the clamping unit. Its curvature is smoothly continued so as to form a seat for the membrane (D) and the O-ring (L) so as to bring bout a tight sealing in conjunction with the membrane itself. The

curvature is designed so that the O-ring is expanding along the major diameter while being clamped ascertaining that the membrane obtains a perfect fit to the supporting surface. The clamping disk (M) has two screws (N) which are tightened by hand and two short guiding plugs to facilitate a smooth operation of the clamping procedure. The hole in the clamping disk on the surface *facing the semipermeable* membrane matches the diameter of the supporting cylinder Towards the other surface the opening is widening so as to form a conical receptacle (P) with a capacity of 1 ml

The clamping unit has a thread (Q) on the cylindric surface of the body to provide for a lucite cover (R) The cover has two small openings (T) (female Luer lock adapters) to permit measurements with samples exposed to various gases and gas mixtures This would also make it possible to operate the instrument as a null indicator if for any reason that should be desired. Tightness is secured by an O-ring (S)

The way the membrane is supported and clamped creates favorable conditions *for the establishment of diffusion equilibrium. The effective reference* volume is reduced to that of the membrane itself and the capillary space beneath it. In the cylindrical space (F) and in the tiny channel (H) there is a concentration gradient to the fluid in the manometric chamber (G) but this gradient does not influence the equilibrium in the effective reference volume and is undisturbed by convexion excepting that brought about by gravitational forces. The design assures that the reference fluid always becomes identical with the ultra filtrate of the sample in the course of a measurement. No *change of* fluid of the pressure chamber other than that resulting from the measuring procedures is necessary The actual effective reference volume is, of course, dependent on the particular membrane used. The membrane which has been found very useful is Schleicher & Schuell, Ultrafine Membrane, Filter Type U A. Dense[1] It is 0 1 mm. thick when wet. The area exposed to the sample is $3.5^2\pi = 38$ mm The maximum volume consequently is about 4 mm³ The volume of the sampl is generally 100 mm³ so that evidently no material change of concentration will ensue as a consequence of diffusion. Even with as small a sample as 20 mm³ which is the smallest recommended no effect on plasma COP *should be expected because* of the difference between the ionic concentrations in the plasma and in 0.9 % saline, assuming no change of pH, an assumption which is justified on the ground of the buffer effect of plasma. This statement is supported by the results of measuring COP in a 5 % or albumin solution with various concentrations of saline (*Fig 5*) The slight effect which is apparent requires much larger deviations in salt concentrations than would ensue in the case of plasma versus 0.9 % saline in the above volume relations. *It is, therefore, safe to use* 0 9 % saline as standard reference fluid. The effect of the salt concentrations is related to the conditions for the *Donnan* equilibrium (WAGNER 1949)

[1] CARL SCHLEICHER & SCHUELL Co., Keene, New Hampshire.

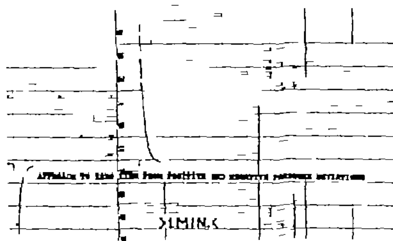


Fig. 4 The figure illustrates the consistency and speed with which the osmometer reverts to zero pressure after deflections in positive and negative direction. Paper speed 15 times that used in routine recordings as displayed in Fig. 5.

Preparation for measurement

Boiling The transducer and the clamping unit are boiled in distilled water to secure complete filling of all paces with air free fluid and for the same eason all parts are assembled under water

Connection to the electric aggregate and testing The osmometer is taken out of the water n such a w y that the water remains in the recess at the bottom of which is the seat for the membrane. The connection to the electric apparatus is established and the performance tested. At this stage the osmometer should be checked with respect to pressure sensitivity even though t stays remarkably stable, uninfluenced by any of the procedures involved in its use.

Pressur alibration. The pressure calibration is carried out by means of the lucite cover (R) in connection with a simple mercury or water manometer and a pressure device delivering both negative and positive pressures. The over-all linearity of the transducer is within 2 from — 300 mm Hg to — 300 mm Hg., and much better than that in the actual range of measuring from zero to — 50 mm Hg Whereas a real pressure calibration is hardly necessary more than once for any transducer it is wise to check the sensitiv ty g every time the membrane is changed and, of course, if the electronic apparatus has been adjusted. One single negative pressure al e will suffice for the calibration besides, of course the zero pressure.

Insertion f the semipermeable membrane is the next step. The membrane is brought to float on the surface of the water left in the recess. The water is sucked away sufficiently much to bring the membrane in touch with the

highest point of the supporting cylinder. The membrane is centered and the O-ring likewise centered put on top of it. The clamping disk is put into position on the body of the clamping unit and the screws are tightened until broad contact is made between the two elements of the clamping unit. During the tightening procedure the pressure is increasing in the osmometer but reverts quickly to the same baseline as was observed with the open osmometer. This serves as a check on the fitness of the membrane and shows that the stopcock is unnecessary to check the baseline (*Fig. 4*). The membrane should never be allowed to dry out pending the clamping procedure and it is to be kept covered by *e. g.* physiological saline between measurements. Distilled water is preferred overnight and in other longer periods of idling for the instrument.

Temperature control

The COP is dependent on temperature according to the *van t Hoff* equation. Ordinary variations in room temperature will, therefore, not disturb the measurement materially. Only if COP is to be measured at other than room temperatures will water bath or similar means to control the temperature be necessary.

However as the baseline for the measurement of COP is temperature dependent too the problem requires a little more consideration. It is safest to place the apparatus in a water bath, but not necessarily to apply temperature control unless measurement at other than room temperature is required or the room temperature is more than usually fluctuating. The temperature must then be kept within approximately ± 0.05 C.

A very practical arrangement that takes care of all possibilities is one in which the osmometer is placed in a commercially available 6 × 6 inches, cylindric stainless steel jar so that the cord to the electric apparatus is entering water tight through a hole made in the bottom. The jar is filled with distilled water to the level of the clamping disk. A stirrer is provided. If other than room temperature is desired water of suitable temperature is run through a coiled-up copper tubing from a temperature-controlled body of water. The arrangement here outlined holds the further advantage that the osmometer may be boiled *in situ*, though partly disassembled, by using an electric dipping heater.

Baseline

The baseline is the level recorded when there is the same osmotic concentration in the effective reference volume as in the receptacle *i. e.* after diffusion equilibrium is established. As the amount of fluid in the receptacle is the same when the baseline is recorded and when the sample is in the receptacle there is no correction to be made, neglecting small differences of density. The baseline is independent of the concentration of the fluid in the receptacle. The zero pressure, when the membrane is removed, is identical with the baseline if care is taken to secure the same hydrostatic pressures in both cases.

The baseline is stable within $\pm$ 0.2 mm Hg for several hours, if the temperature is kept within the limits stated above, and after a couple of hours have been allowed for warming up. Within shorter intervals the variations are even smaller

If the pressure is raised or lowered by turning the clamping unit the pressure will revert to the baseline very neatly as shown in *Fig 4* It will be noted that the approach of the pressure curve to the baseline is symmetrical for positive and negative pressures. It shows that the membrane does not yield more when the pressure is positive inside the manometric chamber than when it is negative, in spite of lacking direct support in the former case. The pressure curves approach zero exponentially in accordance with a Poiseuille type of flow

Displacement of volume

If the semipermeable membrane were absolutely unyielding and the O-ring between the clamping unit and the transducer completely unpliant the volume change per mm Hg would be the same for the osmometer as for the transducer i.e. about 3×10^{-4} mm³ This, of course, is not to be expected.

However a check is easily made by turning the clamping unit a small angle and recording the pressure change. As it can be assumed that only an infinitesimal amount of liquid will have passed the membrane in the short time necessary to turn the clamping unit, the volume/pressure relation will be a valid expression for the elastic properties of the osmometer

The radius of the connecting tube of the transducer is 3 mm and the pitch of the thread 0 75 mm. A full turn is therefore equivalent to a volume change of

$$3^2 \times \pi \times 0\ 75 \text{ mm}^3$$

Averaging three experiments it was found that a turn of about 1° changed the pressure 133 mm Hg The actual figures 6/740 π were obtained by recording the distance (6 mm) travelled by a 370 mm long extended pointer fixed radially to the clamping unit. The volume change per mm Hg is consequently

$$\frac{3^2 \times \pi \times 0\ 75 \times 6}{133 \times 740 \times \pi} \text{ mm}^3 \sim 4 \times 10^{-} \text{ mm}^3$$

It shows that the efforts to secure a rigidly supported semipermeable membrane have been very successful indeed.

Choice of membrane

The performance of the osmometer as described above relates to the use of the membrane mentioned in connection with the diffusion problem. It is a cellulose membrane and as such tougher and more resistant to both mechanical and chemical injuries than the collodion membranes that were recommended in connection with the first version of the osmometer It also saves one the



Fig. 5 A series of routine determinations of osmotic pressure of blood serum. The numbers refer to the marking of the samples examined. The zero is at 80 on the vertical axis, the unit of which is 1 mm Hg. The colloid osmotic pressure is read directly in mm Hg as the difference between 80 and the level of the horizontal part of the upward deflecting. The paper was moving continuously at a speed of 4 inches per hour. One inch is the distance between two heavy vertical lines.

time consuming and often very unrewarding efforts to prepare suitable collodion membranes. However only the further reduction of the volume displacement has made it possible to use a membrane so much thicker. Although most of the membranes from the same batch are usable it pays to pick the most suitable from it. About one third will function perfectly. It is easily seen on the insertion of a new membrane whether it will be quick enough, as earlier mentioned. Whether it is tight enough is tested by leaving a solution of ox albumin or just one of the samples of plasma in the receptacle and observing that the equilibrium position is kept unchanged for an hour. The receptacle must then be covered by e. g. a small beaker which matches the opening of the receptacle in order to prevent evaporation. The membranes have always complied with this test. Hemoglobin has also been used as test material with the same result. The membranes that have been deemed too slow, however, will give the same result with regard to colloid osmotic pressure but the equilibrium will be reached after a longer period of time, though always within about 15 min. The membrane can be used for months without being taken out of the osmometer and there is no change in its properties. As a membrane apparently can stay in the osmometer for a very long time it is very fortunate that the transducer likewise can be left ready for immediate work over at least as long a period of time with no need for re-calibration or re-setting from day to day.

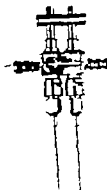


Fig. 6. Combined flushing and suction arrangement. The needle supplied with polyethylene tube is for suction.

The measurement

In the case of plasma or serum the measurement is performed as follows

The conical receptacle of which now the semipermeabl membrane forms the bottom is flushed with 5 to 10 ml. of physiological saline. The remaining saline is removed by suction and the sample deposited in the receptacle by means of a disposable pipette. The exact size of the sample is, of course, immaterial, only it must cover the whole effective part of the membrane. Generally 0.1 ml. is used as mentioned before. The pressure will immediately start to grow negative in the manometric chamber as indicated on the recorder When a plateau is reached after 2 to 4 min the osmotic pressure can be directly read. The next measurement can be made after flushing with saline has taken place. It is not necessary to await a complete return t the baseline, if the temperature control is adequate, and the zero line therefore table. Ten to 15 measurements can be made per hour (*Fig 5*) In accordance with th prediction made in the section dealing with diffusion problems flushing with various concentrations f saline does not affect the colloid osmotic pressure of plasma or serum. However the initial pressure course before equilibrium is attained shows variation in coordance with the different concentrations and the osmotic flow due to the diffusing molecules as already demonstrated in Fig 1 (See the curve for NaCl solution.)

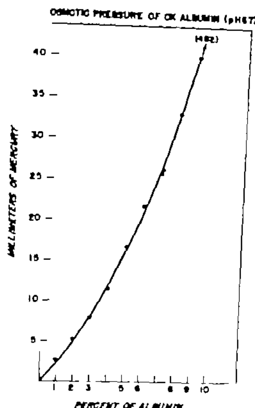


Fig. 7 It is demonstrated here that the osmotic pressure of protein increases out of proportion to the increase of weight/volume concentration. This results (partly) from the fact that the dissolved protein occupies a non-negligible part of the volume of the solution.

Flushing and suction are very conveniently done simultaneously by means of a twin stopcock made from standard units (*Fig. 6*). A 10 ml. syringe is used, filled from a standard saline infusion bottle.

The *confidence limits* of a measurement with all errors compounded is estimated from a series of 10 measurements of 10 consecutive samples from the same serum. Each measurement is carried out exactly as when different sera are used. The limits are ± 0.5 mm Hg for the individual measurement at the 5 % level.

Examples of measurements. A few characteristic results of COP measurements are illustrated. In *Fig. 7* is shown the relations of protein concentration and COP over a range of concentrations comparable with concentrations found in sera. The COP is characteristically rising out of proportion to the protein concentration as this latter is growing when the protein concentration is stated on the usual weight volume basis. The main reason for the lack of proportionality is that the part of the volume of the solution taken up by the protein itself is not negligible as in e.g. salt solutions.

In *Fig. 8* the colloid osmotic pressure is compared with protein concentrations in a number of sera. The COP was measured in the same (not normal) sera in which chemical determination of the protein concentration was carried out

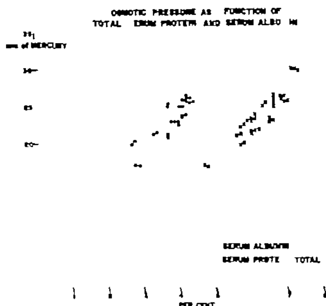


Fig. 8. The osmotic pressure is directly related to the total serum protein values only. The serum albumin values are plotted with the same ordinate as that of the total protein of the sample. The abscissa indicates the actual concentration.

routinely[4] The over-all agreement between the two groups of results is good, but on the assumption that the measurements are carried out correctly it can also be seen that transformation from one quantity to another can only be made with a wide margin of uncertainty in any single case.

Concluding remarks

As stressed throughout th paper the osmometer has been designed with an emphasis on the measurement of COP in body fluids, the assumption being that the pressure measured is the same as that exerted in the organism. The prerequisite would be that the artificial membrane resembles the capillary wall with respect to molecular size discrimination and that no material concentration of protein is found outside the wall. Although none of these pre requisites are entirely fulfilled the over all picture is probably not far from being correct (STARLING 1896 KROGH 1929 LANDIS 1927 DRINKER 1937 PAPPENHEIMER *et al* 1948)

Quite independent of this question the COP measured has, of course its physical significance and the osmometer may be useful for the measurement of osmotic pressure also in other fields and in other ranges of molecular size if suitable membranes used. A possible use will be to follow processes in which

[4] For making the samples available I wish to thank ROY W. BONSNES, PH. D. Chief of Chemistry Laboratory New York Hospital, N.Y.C.

the molecular weight is changed by polymerization or disintegration as a consequence of enzymatic activity

As shown in Fig 1 the difference in molecular size is demonstrated by the transient osmotic effect of penetrating particles. It is reasonable to assume that the osmometer could prove useful in a systematic study of reflection coefficients and their relation to total osmotic pressure (STAVERMANN 1952) In this connection it should be mentioned that the author did *not observe any abnormal* osmosis with various concentrations of urea such as reported in the literature (Grim, 1953)

Instead of using the apparatus for investigating the characteristics of solutions it could be applied to the examination of the properties of membranes. The opportunities for estimating the flow resistance for water is already implicit in the preparation for the ordinary use of the apparatus (see page 10) but could be extended to the testing of other membranes intended for other use.

Attempts have already been made to examine the activity of organic membranes (frog skin) in the apparatus and it is possible that certain aspects of the active and passive transport problems could be elucidated by this technique.

Finally it should be mentioned that the measurement of osmotic pressure of whole blood which was originally anticipated has proved impractical if not impossible. Instead the experiments carried out in this connection have led to interesting observations of the osmotic effect of the erythrocytes themselves under various conditions Results and their possible implications in the living organism have been reported elsewhere (TYBJAERG HANSEN 1961)

I wish to thank M E. MAGNUSSEN, chief instrument maker of the firm O Dich, Copenhagen, who made the *transducer and Mr* NIELS JERNBERG, *instrument design engineer* The Rockefeller Institute Instrument Shop, where the clamping unit was produced.

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From the Department of Pharmacology Karolinska Institutet, Stockholm, Sweden

Attempt to Demonstrate Large Arteriovenous Shunts in Skeletal Muscle during Stimulation of Sympathetic Vasodilator Nerves

By

JOHANNES PIEPER and SUNE ROSELL

Received 27 April 1961

Abstract

PIEPER, J and S. ROSELL, *Attempt to demonstrate large arteriovenous shunts in skeletal muscle during stimulation of sympathetic vasodilator nerves.* Acta physiol. scand. 1961 53, 214—217 — The venous recovery of intra-arterially injected wax spheres of different diameters (average sizes 20μ, 30μ and 40μ respectively) was determined in skinned hind leg preparations from cats. Passage of spheres into the venous out flow would indicate perfusion of large arteriovenous shunts. The results provide no support for th assumption that vasodilator nerve activity opens large arteriovenous shunts of any importance from the quantitative point of view

HYMAN *et al* (1959) concluded that vasoconstrictor and vasodilator nerves to the skeletal muscle vessels may innervate different types of blood vessels and that the vasodilator effect is primarily on non-nutritional vessels. In order to shed further light on this question t has been sought to establish whether large caliber arteriovenous shunts are opened during activity of the sympathetic vasodilator nerves. For this purpose wax spheres of three different average diameters (20μ, 30μ and 40μ) were injected intra-arterially The venous recovery of these spheres during vasodilator nerve activity or under resting conditions was determined. It was assumed that the spheres would not pass through true capillaries. Thus, recovery of spheres in the venous outflow would indicate the existence of perfused arteriovenous shunts of sufficient caliber to allow the passage of the spheres.

Present address Medizinische Forschungsanstalt der Max-Planck-Gesellschaft, Göttingen, Germany

Table I Passage of intra-arterially injected microspheres through the vascular bed of the hind limb muscles of the cat under control conditions and during vasodilator nerve stimulation

Exp. no.	Stimulation = S Control = C	Blood flow		Number of spheres injected			Per cent spheres recovered		
		ml/min	%	20 μ	30 μ	40 μ	20 μ	30 μ	40 μ
1	S	12	100	460	405	420	5	3	2
	S	8	100				4	1	1
	C	4	0				2	1	1
2	S	8	80	230	200	210	2	1	1
	C	7.5	60				0	1	1
	S	8	100				1	0	0
3	S	12	50	290	310	360	2	1	0
	C	6.5	0				1	0	0
	S	8	30				3	1	0
4	S	6.5	90	290	310	360	2	1	0
	C	3.5	0				1	0	0
	S	5	65				1	0	0
5	S	11	110	290	310	360	2	0	0
6	C	2.5	0	205	210	230	0	0	0
7	C	7	0	250	210	230	0	0	0

Per cent above resting level, immediately before injection of spheres.

Methods

The experiments were performed on seven cats weighing 3.5 to 4.5 kg. To eliminate the effects of vasoconstrictor nerve activity reserpine (5 mg/kg) was given subcutaneously 20—24 hours before the acute experiments (ROSELL and ROSÉN 1961). The cats were anesthetized with intravenous urethane (200—400 mg/kg). To prevent clotting, heparin (25 mg/kg) was given i. The trachea was cannulated. The arterial blood pressure was recorded in a common carotid by a Statham pressure transducer (P 23 AA). Blood flow was measured in a hind leg by cannulating the femoral artery and directing the blood through a silicone-filled drop chamber operating an ordinate writer (LINDGREN 1958). The blood re-entered the leg via the cannulated distal stump of the popliteal artery. In order to measure only muscle blood flow the leg was skinned and a tight ligature around the ankle isolated the paw from the circulation. For determination of the number of spheres in the total venous outflow from the muscle region, the distal part of the leg was isolated from the proximal part by mass ligatures just proximal to the knee joint. The only structures left intact were the femur, the nervous supply and the popliteal artery and vein. To maintain warmth and moistness the skin was replaced around the muscle.

The sympathetic chain, isolated and transected via the anterior approach, was stimulated in the distal part with a bipolar silver electrode at the level of L_4—L_5. Supramaximal stimulation produced by a Grass Model S4 stimulator was used throughout.

The recording instrument was a Grass Polygraph.

Injection of Spheres

The spheres were suspended in 2 ml blood from the experimental animal and injected into a closed bypass inserted between the femoral artery and the drop chamber. By shifting the arterial blood flow from the main pathway to the bypass the spheres were injected into the hind leg. The venous blood was collected for a two-minute period, beginning with the injection, by means of a T-cannula inserted in the femoral vein. The number of spheres in the venous outflow was determined by counting them under the microscope (for details see PIIPER and SCHOEDEL 1954).

Results

The results are summarized in Table I. During stimulation of the sympathetic chain the average increase in blood flow was 80 per cent (range 30—110 per cent). The average recovery of injected spheres was 3 per cent of 20μ, 1 per cent of 30μ, and 0 per cent of 40μ spheres. The recovery under control conditions did not differ significantly from that during vasodilator nerve activity. The recovery of spheres was in all cases low.

Discussion

The microsphere injection method has been appraised by PIIPER and SCHOEDEL (1954) and PIIPER (1957). Recovery of intra-arterially injected spheres in the venous outflow is thought to demonstrate the existence of perfused arteriovenous communications of larger caliber than the diameter of the spheres. However, the method provides no information on the morphologic or functional characteristics of such vessels, i. e., no evidence as to whether they are thin-walled permeable capillaries, or thick walled impermeable arteriovenous shunts. In most cases studied, the passage of 30μ and 40μ spheres appeared to indicate the presence of shunt flow, whereas in some instances the 20μ spheres were apparently able to pass through capillary vessels (PIIPER 1957). Since in the present study the recovery fraction was very small for all spheres, the question as to the type of vessels through which the different spheres passed is immaterial. The very low recovery fraction of the spheres is assumed to indicate the absence of significant perfusion of large-caliber arteriovenous communications both under control conditions and during vasodilator stimulation.

DIETER (1954), using the same microsphere injection method in experiments on the isolated gastrocnemius muscle of the dog, found a considerably higher recovery fraction of intra-arterially injected microspheres: 17.3 per cent of 20μ, 4.3 per cent of 30μ, and 1.2 per cent of 40μ spheres. He was unable to determine whether the relatively high recovery fraction of 20μ spheres was attributable to perfused arteriovenous anastomoses of relatively small caliber to large capillaries, or to great distensibility of capillaries in the relevant preparation. The quantitative discrepancy between our results and those of DIETER might be due to species differences.

The results of HYMAN *et al.* (1959) indicated that the increased blood flow elicited by stimulation of the vasodilator pathway might pass through non-nutritional vessels, since the tissue clearance of radioactive iodide remained unchanged. Our findings do not preclude the possibility that the increased blood flow traversed non-nutritional arteriovenous channels or "functional shunts" they merely suggest that the caliber of such shunts was smaller than 20 μ. Such arteriovenous communications of small diameter and short length, and hence of low resistance, have been reported in skeletal muscle by ZWEIFACH (1937). According to SAUNDERS (1957) arteriovenous shunts of large caliber occur in the vascular bed of human skeletal muscle. The main question is, however, whether such shunts are of any quantitative significance. To judge from our results they are quantitatively negligible, at least under "resting" conditions and during vasodilator nerve activity.

The aim of this study was to ascertain whether the increase in muscular blood flow elicited by stimulation of the vasodilator pathway passes through arteriovenous communications of large caliber (greater than 20 μ). Our observations on the skinned hind limb preparations afford no evidence of the opening of large arteriovenous channels during vasodilator nerve stimulation.

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From the Institute of Neurophysiology University of Copenhagen, Denmark

Electrical Signs of the Relation between Caudate Nucleus and Cerebral Cortex in Cats[1]

By

ARNE MOSFELDT LAURSEN

Received 15 May 1961

Abstract

LAURSEN, A. MOSFELDT. *Electrical signs of the relation between caudate nucleus and cerebral cortex in cats.* Acta physiol. scand. 1961. 53. 218—232. — Cortico-caudate and caudato-cortical relationships were explored in the cat using electrical stimuli and recording with gross and micro-electrodes. The difference in method from previous studies consisted in the use of a 14-lead multilead electrode.

A. *Caudate stimulation and recording from the cortex*

1) A potential elicited in the caudate by stimulation of the caudate was spread to the cortex by volume conduction.

2) Potentials arising in the cortex in response to single shock stimulation of the caudate were not found.

3) Cortical recruiting responses were only elicited from the internal capsule and the thalamus and from a fringe of tissue of the head of the caudate bordering on these structures.

Spread of current from the commonly used stimulating electrodes is thought to explain the contrary results of other authors.

B. *Cortical stimulation and recording from the caudate*

1) No gross response could be elicited in the caudate by stimulation of the cortex.

2) Single units in the caudate responded to single shock stimulation of the ipsilateral motor cortex.

These results confirm cortico-caudate connections, but make even indirect caudato-cortical connections hypothetical.

[1] A preliminary report was presented in Dan. Med. Bull., 1958, 5 166—167

There is abundant anatomical evidence of cortico-striate connections (MARINESCO 1895 RAMON Y CAJAL 1909—1911 MINKOWSKI 1923 KARIYA 1936, HARRAWA 1936 GLEES 1944) In agreement with this evidence corticostriate connections were found by the method of strychnine neuronography in macaque (DUSSER DE BARENNE and MCCULLOCH 1938, DUSSER DE BARENNE, GAROL and MCCULLOCH 1942) and chimpanzee (GAROL and MCCULLOCH 1944) These findings have been confirmed in the cat (ALBE FESSARD OSWALDO-CRUZ and ROCHA MIRANDA 1960 a) and in addition electrical stimulation of the cortex was found to evoke gross responses (ALBE FESSARD *et al.* 1960 a) and responses of single cells (ALBE FESSARD *et al.* 1960 b) in the head of the caudate.

On the other hand, with the exception of one report (HARMAN *et al.* 1954) there is no anatomical evidence of direct caudate-cortical connections (VONEIDA 1960) nor were such connections found by strychnine neuronography (DUSSER DE BARENNE and MCCULLOCH 1938) It has been reported that cortical spindle bursts may be elicited by stimulation of the head of the caudate in cat (JUNG and TÖNNIES 1950 SHIMAMOTO and VERZEANO 1954 UMBACH 1959 WIECK *et al* 1960) This response was abolished by destruction of the diffuse thalamic projection system (SHIMAMOTO and VERZEANO 1954) a finding consistent with th anatomical evidence (RANSON, RANSON and RANSON 1941 PAPEZ 1942, WOODBURNE, CROSBY and MCCOTTER 1946, GLEES 1945 JOHNSON and CLEMENTE 1959) if the nucleus ventralis anterior and lateralis were included in the lesion. Cortical recruiting responses (DEMPSEY and MORISON 1942) were found to be elicited from the head of the caudate (AJMONE MARSAN and DILWORTH 1953 STOUPEL and TERZUOLO 1954 WIECK *et al.* 1960) but in this case too, a direct caudate-cortical pathway seemed to be excluded When cortical seizure activity was inhibited by single shocks to the caudate (UMBACH 1959) the effect was thought to be mediated by the intralaminar thalamic nuclei. PURPURA, HOUSEPIAN and GRUNDFEST (1958) and WIECK *et al* (1960) reported short latency potentials in pericruciate cortex of cat evoked by stimuli applied to the head of the caudate, indicating direct caudate-cortical pathways.

I am reporting a study in which cortico-caudate and caudate-cortical relationships were explored using electrical stimuli and recording with gross and micro-electrodes. The difference in method from previous studies consisted in the use of a multilead electrode (BUCHTHAL, GULD and ROSENFALCK 1957) allowing well defined changes in the sit of recording and of stimulation and evaluation of the extent to which structures other than the caudate were stimulated and might ha e been responsible for the effects.

Method

Material. Cats weighing from 2 to 4 kg were used. Sixteen were anesthetized with a 1 per cent solution of chloralose injected intraperitoneally in doses averaging 80 mg/kg and 11 were prepared as encéphale isolé under ether anesthesia. Wounds and pressure points were locally anesthetized with tetracaine. Three cats were prepared as cerveau isolé by an intercollicular section and in only one of these could recording be carried out without interference from cerebral edema. Finally in four cases the animal was curarized (LAUDOLISSIN® ALLEN and HANBURY Ltd.) after wounds and pressure points had been locally anesthetized with tetracaine and the operation performed under ether.

Encéphale isolé. These preparations were studied in the sleeping as well as the aroused state, the sleeping state being characterized by the occurrence of spindles and slow waves in the EEG and arousal by low voltage fast activity. Preparations which did not show clear behavioral response on pinching the ear were discarded. When cortical responses were to be recorded under conditions of arousal this state was maintained by stimulation of the mesencephalic reticular formation through a concentric electrode using rectangular pulses of 1 msec duration and frequencies of 50 to 200 per second.

Caudate stimulation. The multilead electrode used for stimulation and recording in the area of the caudate nucleus was modified from the multilead electrode described by BOCHTHAL, GULD and ROSENFALCK (1957). For use in the central nervous system it contained 14 platinum leads each 0.1 mm in diameter distributed along 5 mm of a stainless steel cannula with an external diameter of 1 mm. The distance between the centers of adjacent leads was 0.4 mm. The electrode was stereotactically inserted into the exposed brain and in the different experiments a series of angles and planes of insertion was chosen so as to make a thorough exploration of the head of the caudate nucleus. To minimize the damage produced by the electrode and to make it possible to determine its position after the experiment, only one insertion was made in each brain. Stimuli were applied through different pairs of adjacent leads.

For comparison the caudate nucleus and adjacent structures were explored with the following stimulating electrodes: a) Concentric electrode, 0.7 mm in diameter with central leading off area of $100 \times 140\ \mu$. b) Bipolar electrode, 0.7 mm in diameter with two $100 \times 140\ \mu$ leading off areas. The distance between the centers of the leads was 0.5 mm. c) Unipolar electrode made of a steel needle, 0.8 mm in diameter including two coats of laquer with 0.5 mm bared at the pointed tip.

To reduce artefact the rectangular stimuli of 0.1 to 1 msec duration were applied via a double screened transformer (BUCHTHAL, GULD and ROSENFALCK 1955). Stimulating currents were of the order of 0.2 to 2.5 mA.

Cortical stimulation. Stimuli were applied to the cortex through two steel needle electrodes 2 to 4 mm apart.

Recording from the caudate. a) When the multilead electrode was used for recording from the caudate the following arrangements were tested: i) Leading off between pairs of adjacent leads. ii) Leading off between the different leads and the cannula of the multilead electrode. iii) Leading off between one lead of the multilead electrode and a screw inserted in the skull over the frontal sinus. b) Unit potentials were recorded through glass micropipettes filled with 3 M NaCl with impedances of 5 to 10 MΩ. The electrode was advanced by an automatic microelectrode transport device (ANDERSEN and LAURSEN 1959) installed in the roof of a chamber cemented to the skull (DAVIES 1956) and was inserted into the caudate through the exposed intact brain from its dorsolateral surface.

Recording from the cortex. The following types of recording were used: a) To reveal local potentials or potentials spread by volume conduction from the depth, recording was carried out from two symmetrical points on both hemispheres through spring loaded

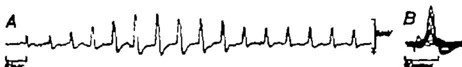


Fig. 1 Thalamically evoked recruiting responses recorded continuously (A) and as superimposed traces (B)

silver ball electrodes resting on the pia. The common indifferent electrode was a screw in the skull over th frontal sinus. Before response was recorded the indifferent electrode was tested against a sil er needle in the temporal muscle to ensure that it did not record responses. b) When studying cortical evoked potentials under conditions of arousal maintained by reticular stimulation it was necessary to eliminate the artefact produced by the reticular stimulation from the record. This was done by leading off between two pial electrodes whose position was shifted until no artefact from reticular stimulation was recorded.) To study the distribution of evoked changes in the electrocorticogram 6 silver ball electrodes were cemented into holes in the skull in contact with the dura. The indifferent lead was screw in the skull over the frontal sinus.

When recording was from the pia the dorso-lateral surface of the cortex was exposed bilaterally and covered with pool of mineral oil. The temperature was measured with a thermistor in contact with the cortex and maintained at 37° C.

Recording apparatus. The electrocorticogram was recorded with 6 channels of an electroencephalograph. Evoked responses recorded with gross electrodes were amplified via differential amplifier (GOLD)[1] with frequency response of 3 db down at 10,000 and 1 c/sec. The signal from the microelectrode was led through cathode follower to the same amplifier The cathode follower added 2 μμF to the capacity of the electrode and input cable. The frequency response employed was 3 db down at 10 000 and 100 cps. The responses from symmetrical points of the two hemispheres were simultaneously displayed on a two-beam oscilloscope. T distinguish evoked gross responses from spontaneous activity 5 to 15 traces were superimposed on the photographic film. The ppearance of recruiting response in this type of recording is shown in Fig. 1 B. Unit responses were recorded using single sweeps triggered from the stimulator

Determination of electrode positions. T determine the sit of the multilead electrode relative to macroscopic structures the heads were perfused with the electrode *in situ*. The perfusing fluid consisted of 5 per cent formalin in Ringer' solution with 5 per cent added colloid (dextran) It had been found that the shrinkage of brain slices in this fixative was about 3 per cent (Knappen) After fixation the calvarium was removed and the brain was sliced parallel to the axis of the electrode exposing the leads. The tips of the other stimulating electrodes were marked by the method of MARSHALL (1940) by passing current from the core of the concentric or from one of the inner leads of the bipolar electrode to large electrode placed in the rectum. The brains were prepared for histological examination so that the position of each stimulation point could be calculated. The tracks of the macroelectrodes were located by histological examination of the brains which had been perfused and fixed with the microelectrode *in situ*. Paraffine sections, cut t 20 μ and stained with thionine were used.

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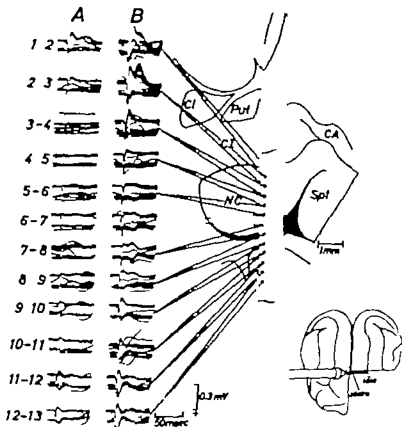


Fig. 2. Chloralose anesthesia. Responses to stimulation of the caudate and surrounding structures, recorded from symmetrical points of the two anterior sigmoid gyri using common indifferent electrode. The upper tracing of each pair was recorded ipsilateral, the lower contralateral to the side of stimulation. Fifteen superimposed sweeps. Column A shows responses to 1 msec stimuli slightly above threshold for cortical afferents at 1/sec. Column B shows responses to stimuli at twice the intensity. Weak stimulation between leads 1—2 and 2—3 elicited an early positive-negative response and later negative recruiting response. Weak stimuli between leads 9—10 10—11 11—12 and 12—13 elicited callosal response. Strong stimuli between leads 5—6 6—7 7—8 and 8—9 evoked local caudate responses which are spread by volume conduction (see text) to the cortex and appeared as mirror images in ipsi- and contralateral records. Cortical responses to stimulation in the caudate were otherwise absent. CA Commissura anterior CI Capsula interna. NC Nucleus caudatus. Pu Putamen. Spt Area septalis.

Results

A. *Caudate stimulation and recording from cortex*

1 *Single shock stimulation*

Multilead electrode inserted through the caudate nucleus and adjacent structures Whether the animal was anesthetized or not single pulses delivered to the caudate did not evoke cortical potentials unless the strength of the stimulus was several times the threshold for cortical responses to stimuli delivered to the internal capsule (Fig 2) The source of the potential which was evoked from the medial

part of the caudate by pulses exceeding this strength (Fig 2 column B, leads 5—9) was not situated in the cortex as it was not altered in any respect by local cortical anesthesia or ablation That it was elicited from the caudate nucleus was demonstrated by the fact that removal of the larger part of that nucleus through a small temporal exposure abolished the potential. The potential was thus spread by volume conduction from the caudate to the cortex. The shape of this potential depended on the site of recording On the ipsilateral hemisphere (Fig 2, upper of each record pair) the response had a latency of 10 msec and consisted of a positive deflection followed by a longer lasting negative phase. On the contralateral hemisphere (Fig 2, lower of each record pair) the shape of the potential was the mirror image of this. The amplitude of the response decreased as the recording sites were moved occipitally

By stimulation of points adjacent to the caudate cortical potentials were elicited which disappeared after local cortical anesthesia or ablation and must thus be considered to arise in the cortex itself. Stimulation of cortical afferents in the internal capsule evoked a diphasic response with the first phase positive in the ipsilateral sensorimotor cortex (Fig. 2, column A, leads 1—3 column B leads 1—5) Stimulation of the corpus callosum produced a response (Fig. 2 B leads 8—13) which could be identified by its bilateral, symmetrical occurrence. Its change from an early positive deflection to a diphasic, positive negative potential, when the strength of stimulation was increased is in agreement with the description given by CURTIS (1955 Fig 4 records J—R)

Bipolar electrode used for exploration of the caudate nucleus. Stimulating through the bipolar electrode the cortical potential due to volume conduction from the caudate was absent, possibly because the stimulating leads at the tip of the electrode were not oriented properly in relation to the ventricular surface of the caudate.

Concentric electrode used for exploration of the caudate nucleus. Stimulation of the caudate evoked cortical potentials whose latency shape and amplitude depended on the location of the electrode and the strength of stimulation. No attempt was made to analyse these potentials because the extent to which they were elicited by current spread to other structures was difficult to evaluate.

2. *Repetitive stimulation at rates of 1—15/sec*

Multilead electrode inserted through the caudate nucleus and adjacent structures

i) With leads in the caudate, recruiting potentials were seen in ipsilateral cortex when the lead pairs were in the head of the caudate immediately adjacent to the internal capsule (Fig 2, lead pairs 1—2 and 2—3 upper of the record pair) Cortical recruiting responses were absent when stimulation was carried out through lead pairs situated in the rest of the caudate explored (Fig 2 Fig. 3)

ii) With leads in the thalamus, recruiting responses were evoked in the cortex (Fig. 3 Fig 4 With lead pairs near the midline (Fig. 3 lead pairs 1—2) the

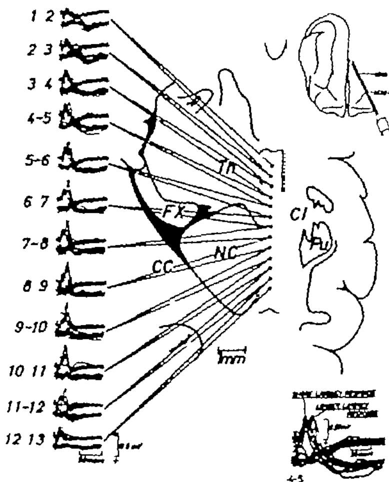


Fig. 3. Encéphale isolé. Recording as in Fig. 1. Repetitive stimulation at 7/sec. The response to stimulation through lead pairs close to the midline (leads 1—2) was bilateral and gradually became ipsilateral only as the stimulus was moved away from the midline in a rostrolateral direction. CC Corpus callosum. CI Capsula interna. F Fornix. NC Nucleus caudatus. Pu Putamen. Th Thalamus.

cortical recruiting response was bilateral. As the site of stimulation was moved in a rostrolateral direction the response became more purely ipsilateral (Fig. 3). This characteristic of the thalamic recruiting system was studied by Enomoto (1959) and the relevant literature is reviewed by him. The recruiting system extended continuously from the thalamus through the internal capsule to opposite the rostral pole of the caudate (Fig. 3).

iii) With lead pairs in the internal capsule, recruiting responses were evoked in ipsilateral cortex (Fig. 3).

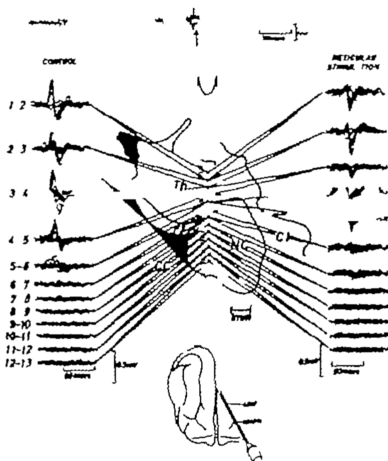


Fig. 4. Encéphale isolé. Recording between two pial electrodes on the anterior suprasylvian gyrus on the side where the caudate and thalamus were stimulated at a frequency of 7/sec. Fifteen superimposed sweeps. The column of responses marked control was recorded during the presence of the electrocortical sleep pattern demonstrated in the left half f the top record. The column marked reticular stimulation was recorded during the arousal response illustrated in the right half of the top record. Arousal was maintained by stimulation of the mesencephalic reticular formation through concentric electrode using 1 msec pulses at frequency of 215/sec. Arrow marks onset of reticular stimulation. CC Corpus callosum. CI Capsula interna. F Fornix. NC Nucleus caudatus. Th Thalamus.

It was found by STOUPEL and TERZUOLO (1954) and confirmed in this study that recruiting responses elicited from rostral areas have short latencies. The experiment illustrated in Fig. 3 showed that the variation in latency in a rostro-caudal direction was not a gradual increase but that two sets of recruiting responses with different latencies were evoked in the cortex. From a rostral area in the internal capsule responses were evoked with stimulus peak times of 7

10 msec. From a more caudally situated area, overlapping with the former responses were evoked with stimulus peak times of 16—21 msec.

The optimum stimulus frequency was 7/sec for both groups of recruiting responses. It was lower in anesthetized than in unanesthetized animals (compare Fig. 2 with Figs. 3 and 4) and often decreased as the experimental time increased.

Bipolar electrode. Experiments in which this stimulating electrode was used for exploration of the head of the caudate and adjacent structures gave the same results as those obtained with the multilead electrode.

Concentric electrode. In contrast to the results obtained with the multilead and bipolar electrodes, stimulation through the concentric electrode evoked cortical recruiting responses from the head of the caudate as well as from the internal capsule and thalamus. More spread of current from the concentric electrode was assumed to be the reason for the contrasting results and an attempt was made to measure thresholds for cortical recruiting responses because these were expected to be higher in the caudate than in the internal capsule and thalamus. However, the thresholds varied so much that no significant difference could be ascertained.

B. *Caudate stimulation and recording from cortex combined with reticular stimulation*

Cortical desynchronization evoked by stimulation of the mesencephalic reticular formation is characterized by diffuse cortical facilitation as shown by the increase of the evoked potential recorded in the cortical receiving area when a stimulus is applied to the corresponding thalamic relay nucleus (BARDIER and STOUPEL 1959). This is the case in spite of the fact that cortical recruiting responses (MORUZZI and MAGOUN 1949) as well as peripherally evoked sensory responses in the cortex (GAUTHIER, PARMA and ZANCHETTI 1956) are depressed during arousal.

The possibility that cortical responses to caudate stimulation would appear only during arousal was investigated by exploring the head of the caudate with single shocks and slow repetitive stimulation delivered through the multilead electrode, while attempts were made to record potentials from the cortex. Cortical arousal was maintained by stimulation of the mesencephalic reticular formation at 200/sec. No cortical responses were found (Fig. 4, leads 6—15).

Recruiting responses elicited from the thalamus and recorded between two electrodes resting on the pia exhibited changes in shape during arousal in addition to the expected reduction in amplitude (Fig. 4, leads 1—6). No analysis of the mechanism of this phenomenon was made.

C. *Cortical stimulation and recording from the head of the caudate*

1. *Gross responses*

The dorsolateral surfaces of the hemispheres were explored with the stimulating electrode using frequencies from single stimuli with 2—3 sec intervals to

200 sec and strengths up to 20 V while attempts were made to detect an electrical response at the individual leads of the multilead electrode situated in the head of the caudate. The result of the search was negative in contrast with the findings of ALBE-FESSARD *et al* (1960 a). The discrepancy may be due to the systematic use by these authors of at least 3 sec intervals between stimuli in animals anesthetized with chloralose and 10 to 20 sec intervals in unanesthetized preparations.

2. *Unit responses*

It was confirmed that single units in the head of the caudate respond to single shock stimulation (2 to 3 sec intervals) of the ipsilateral motor cortex (ALBE-FESSARD *et al* 1960 b) in unanesthetized cats. Stimulating at constant intensities just above those required to produce movement (as determined prior to curarization) one or two spikes occurred the first with a latency of between 15 and 45 msec. Areas outside the ipsilateral motor cortex were not explored.

ALBE-FESSARD *et al.* (1960 a) found very short latencies for caudate gross responses elicited from psilateral motor cortex and inferred a direct pathway The latency of unit responses found by these authors (ALBE-FESSARD *et al* 1960 b) was of the same order (averaging 27.5 msec) as that found in this study

Discussion

Cortico-striate connections demonstrated by strychnine neuronography (from areas 4 s, 8, 24 and 2) in macaque (DUSSER DE BARENNE and MCCULLOCH 1938 DUSSER DE BARENNE, GAROL and MCCULLOCH 1942) and chimpanzee (GAROL and MCCULLOCH 1944) have been confirmed in cat. Here they arise from bilateral motor cortex and sensory cortex I and II of both sides (ALBE FESSARD *et al.* 1960 a, b) and were confirmed from ipsilateral motor cortex in this study Direct connections in the form of unmyelinated collaterals from cortico-fugal fibers were found histologically (GLEES 1944) and direct connections were also indicated by the short latency of gross responses evoked in the caudate from the motor cortex (ALBE FESSARD *et al* 1960 a) The long latency of unit esponses found by ALBE FESSARD *et al* and in this study has been attributed by her to transmission in the caudate.

On the other hand the existence of direct caudate-cortical connections is very doubtful. Recently VONEIDA (1960) using the Nauta-Gygax silver impregnation technique found no degeneration n the cortex after lesions in the head of the caudate in monkeys and cats. In the study reported here neither responses evoked by single shocks nor recruiting responses (as originally described by MORISON and DEMPSEY (1942)) could be elicited by electrical timulation confined

to the head of the caudate nucleus. It is true that only sporadic attempts were made to elicit "spindle tripping" (as originally described by DEMPSEY and MORISON (1942)). Failure to find cortical responses to stimulation of the caudate was not due to damage produced in it by the multilead electrode because i) Cortical responses were obtained from areas outside the caudate where the damage produced by the multilead electrode was the same. ii) The negative results were confirmed using a bipolar stimulating electrode of small diameter and conventional design. Spread of current from the commonly used concentric electrodes were thought to explain the results reported by other authors (AJMONE MARSAN and DILWORTH 1953, STOUPEL and TERZUOLO 1954, PURPURA, HOUSEPIAN and GRUNDFEST 1958, WIECK *et al.* 1960).

Spread of current from the multilead electrode is not less than from a conventional bipolar electrode. The advantage of the multilead electrode is the well-defined mutual localization of the leads which permits comparison of excitability of different locations in the brain without movements of the electrode. Moving the bipolar electrode through the brain introduces a source of error because the soft and sticky brain tissue may move along with the electrode in unpredictable steps.

A reservation must be made. Stimulation of the fringe of tissue of the head of the caudate bordering on the internal capsule and thalamus did elicit cortical recruiting responses. This may indicate current spread within small distances even when stimulation was between small electrodes 0.4 mm apart (multilead and bipolar electrodes). Or it may indicate the presence of unspecific thalamo-cortical fibers in the part of the caudate adjoining the internal capsule. Such fibers have been shown to ascend in the internal capsule in close proximity to the head of the caudate (NASHOLD, HANBERRY and OLSZEWSKI 1955) and the border between internal capsule and caudate is not sharply demarcated.

Some comment on the study by PURPURA, HOUSEPIAN and GRUNDFEST (1958) is necessary because these authors recorded from the medullary pyramids as a measure to detect spread of current to the internal capsule. PURPURA *et al.* found short latency potentials in the ipsilateral pericruciate cortex of the unanesthetized cat on single shock stimulation of the head of the caudate. The potentials were initially positive and either polyphasic or diphasic of long duration. Longer latency, long duration responses were occasionally observed in the lateral gyrus.

The pyramidal monitoring used by PURPURA *et al.* as a precaution against spread of current is insufficient because: a) Spread to the internal capsule would be revealed by pyramidal monitoring only if the threshold of cortical afferents were the same as or higher than that of the efferent fibers. b) In addition to the internal capsule, corpus callosum and thalamus, notably its nucleus ventralis anterior (HANBERRY and JASPER 1953), are areas to which current from an electrode in the caudate may spread, eliciting a variety of simple and mixed cortical responses.

If direct caudate-cortical connections do not exist two questions arise: a) Does the caudate influence cortical activity? And in case it does b) By which subcortical relay?

a) The experiments reported here showed that neither cortical responses to single shocks nor cortical recruiting responses were in fact evoked from the caudate. Laursen (1961) found that electro-cortical arousal could not be elicited from the head of the caudate. It is possible that also cortical burst tripping was in fact elicited from areas outside the caudate. Further evidence for caudate influence on cortical activity was the "suppression" produced in areas L4s and A4s of the monkey by strychninization of the head of the caudate (Dusser de Barenne and McCulloch 1938). However the description of the suppression corresponds in this case to the spreading cortical depression of Leao (1944). Umbach (1959) described inhibition of cortical seizure activity elicited by single shocks to the caudate. The results obtained by this author are difficult to interpret because he used stimulating electrodes with up to 3 mm interelectrode distance and stimulating currents from 2.5 to 25 mA (approximately 10 times the currents used in the experiments reported here).

b) Even if caudate influence on the mentioned types of cortical electrical activity is questionable, such influence in a broader sense remains a reasonable hypothesis. The recently demonstrated caudate participation in processes of learning (Rosvold and Delgado 1956, Rosvold, Mishkin and Szwarcbart 1958, Bättig and Rosvold 1959, Dean and Davis 1959, Thompson 1959) would otherwise be difficult to understand. Recently Voneida (1960) showed that caudate outflow is limited to the globus pallidus and the substantia nigra in cat and monkey (macaque). Signals could be relayed from the globus pallidus to the cortex via nucleus ventralis anterior and lateralis of the thalamus (Papez 1942, Glees 1945, Woodburne, Crosby and McCotter 1946, Johnson and Clemente 1959, Umbach 1959). The optic thalamus was a relay for the cortical suppression elicited from the caudate by Dusser de Barenne and McCulloch (1938).

In this study a response evoked in the caudate by stimulation of the caudate was recorded from the cortex to which it had spread by volume conduction. Local caudate potentials have been reported previously by Umbach (1959) and by Purpura.

Umbach found local spindles preceded by a "Vorwelle" and a "Hauptwelle" in response to single shocks to the caudate but for reasons mentioned above it is difficult to evaluate his results. Purpura stimulated the exposed ventricular surface of the caudate and evoked a focal, negative potential, similar to the response recorded in this study.

In concluding I wish to point out that the caudate in relation to the recruiting response behaves as the cerebral cortex. Recruiting responses can be evoked in both of these telencephalic areas as well as in the thalamus by stimulation of

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the thalamus (Verzeano, Lindsley and Magoun 1953) but recruiting responses cannot be evoked by stimulation confined to the telencephalon. It may be significant in this connection that the morphological substratum for the synapse in the striatum appears to be predominantly represented by a pericellular plexus very similar to the pericellular plexuses of the cerebral cortex, in which terminal boutons, if present, are very few in number (Bielschowsky 1919 Glees 1944)

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From the Institute of Neurophysiology, University of Copenhagen, Denmark

Caudate Nucleus and Electrocortical Activation in Cats

By

Arne Mosfeldt Laursen

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Abstract

Laursen, A. Mosfeldt. *Caudate nucleus and electrocortical activation in cats.* Acta physiol. scand. 1961. 53. 233—238. — A 14-lead electrode was used to search for areas in and around the caudate nucleus from which electrocortical arousal could be elicited. Electrocortical arousal was induced from the reticular ucleus of the thalamus and the internal capsule and, probably by current spread, from the fringe of caudate tissue adjacen to the thalamus, but not from other portions of the caudate. The contrary results of other authors were ascribed to spread of current from the commonly used stimulating electrodes. It is concluded that there is no evidence to support the iew that the caudate is part of, or closely related to, the ascending reticular activating system.

The aim of the study presented this eport was to determine whether the electrocorticogram could be activated by electrical stimulation of the head of the caudate nucleus in cats.

Previous findings are contradictory. Gerebtzoff (1941) found that cortical activation-desynchronization could be induced from the striatum. Starzl, Taylor and Magoun (1951) in a study of the diencephalic extension of the reticular activating system, stated that the internal capsule rather than the basal ganglia appeared to be the excitable focus. Later investigations (Shimamoto and Verzeano 1954, Stoupel and Terzuolo 1954) agreed with Gerebtzoff that electrocortical arousal could be elicited by stimulation of the caudate nucleus.

In a previous study it was shown that cortical potentials which had been ascribed to stimulation of the caudate were in fact elicited from areas outside the caudate (LAURSEN 1961). Spread of stimulating current might also be responsible for electrocortical arousal induced from the caudate. I am reporting a study in which multilead stimulation technique was used to delineate areas in the region of the head of the caudate from which electrocortical arousal could be obtained.

Material and Method

Eleven healthy mature cats weighing between 2 and 5 kg were prepared as encéphale isolé under ether anesthesia, five by an open operation and six by intraspinal injection of 0.1 to 0.2 ml of tetracaine or alcohol at the level of C. 1. Wounds and pressure points were locally anesthetized with tetracaine.

Two types of stimulating electrodes were used.

a) A 14-lead electrode (BUCHTHAL, GULD and ROSENFALCK 1957) adapted for use in the brain (LAURSEN 1961). The diameter of the electrode was 1 mm, the diameter of the leads was 0.1 mm and the distance between the leads was 0.4 mm. Stimulation was carried out between pairs of adjacent leads. The method of insertion of the multilead electrode and the method for determination of its position have been described (LAURSEN 1961).

b) A monopolar steel needle electrode was used for comparison. Its diameter was 0.8 mm including two coats of laquer. Its pointed tip was bare due to shrinkage of the laquer during hardening at 60° C for 12 hours. A clip in the edge of the wound was used as indifferent electrode. The needle electrode was inserted stereotactically in the brain and its position was determined after the experiments by the method of MARSHALL (1940). The brains were prepared for histological examination using frozen sections cut at 20 μ and stained with thionine.

To reduce artefact, the rectangular pulses of 1 msec duration and frequencies from 50 to 300/sec were delivered through a double screened transformer (BUCHTHAL, GULD and ROSENFALCK 1955). The stimulating currents were varied between 0.2 and 2.5 mA.

Two types of recording were used.

a) To study the electrocorticogram (EEG) during stimulation it was necessary to use a recording system with a sufficiently high frequency response to ensure detection of low voltage artefact from 150—200/sec stimulation. High gain difference amplifiers (GULD)[1] with a frequency response down 3 db at 10,000 and 1/sec were used and the signals were displayed on one beam of a two beam cathode ray oscilloscope the other beam being used for marking of the stimulus. The standing spots were photographed on moving film. Stimulus artefact was eliminated from the record by leading off between two silver ball electrodes resting on the pia in the positions which were found to give least interference. The exposed brain was covered with a pool of paraffine oil and maintained at a temperature of 37° C.

b) To study the distribution of evoked changes in the EEG, six silver wires 0.5 mm in diameter in contact with the dura, were cemented into holes in the skull and connected to an electroencephalograph using a screw in the skull over the frontal sinus as indifferent electrode.

[1] In preparation.

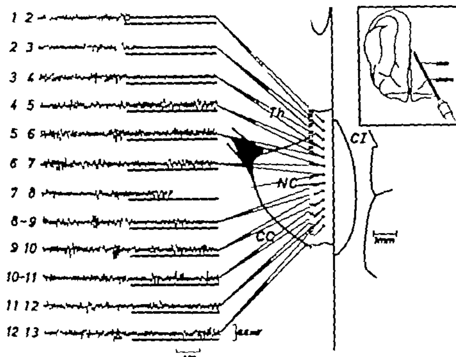


Fig 1 *Absence of electrocortical arousal during electrical stimulation of the caudate nucleus through multilead electrode*

Cat, encéphale isolé. Bipolar recordings (oscilloscope record) from superior suprasylvian gyrus ipsilateral to the side of insertion of the multilead electrode. One msec stimuli were delivered through pairs of adjacent leads at frequency of 200/sec. The black lines under the records indicate stimulation. Electrocortical arousal was produced by stimulation through lead pairs 1—2, 2—3 and 3—4 situated in the reticular nucleus of the thalamus and the fringe of the caudate adjoining it and not by stimulation through lead pairs 4—13 situated elsewhere in the caudate.

CC Corpus callosum. CI Capsula interna. NC Nucleus caudatus. Th Thalamus.

Results

The head of the caudate nucleus and adjacent structures were explored with the multilead electrode, making only one insertion in each brain to minimize damage.

Low voltage, fast activity was elicited in the EEG during and after 200/sec stimulation through lead pairs situated in the reticular nucleus of the thalamus (Fig 1 leads 1—2, 2—3) A less pronounced effect was evoked by stimulation through lead pairs situated in a fringe of tissue of the caudate bordering on the thalamus (Fig 1 leads 3—4) and the effect was probably due to current spread to the thalamus. Stimulation through lead pairs situated in the rest of the caudate did not change the sleep pattern of the EEG (Fig 1 leads 4—13)

When the multilead electrode was inserted in a frontal plane so that the



Fig. 2. Electrocortical arousal produced by stimulation of the right internal capsule through the multilead electrode (lead pair 8—9. 1 msec pulses at 150/sec indicated by black line)

An identical response was elicited by stimulation through lead pair 7—8. Stimulation through the other lead pairs did not elicit electrocortical arousal. Cat encéphale isolé. Extradural unipolar recording. Recording sites shown to the left. Stimulation sites shown on the photograph to the right. The strength of stimulation was 2.5 times the threshold for ipsilateral pericruciate arousal. The flattening outlasted the stimulus by 40 sec and was most pronounced in the ipsilateral pericruciate region, slightly less pronounced in the contralateral pericruciate region and just detectable in the other leads.

CC Corpus Callosum. CI Capsula interna. NC Nucleus caudatus. Spt Area septalis

internal capsule was explored as well as the caudate, electrocortical arousal was elicited only by stimulation through lead pairs situated within a small region in the internal capsule (Fig. 2 leads 7—8, 8—9). Threshold stimulation (approximately 0.3 mA) aroused the EEG in the ipsilateral pericruciate region. With progressively increasing stimulus intensity also the contralateral pericruciate region and the rest of the cortex were activated.

The failure to find electrocortical arousal during stimulation of the head of the caudate was not due to damage produced in it by the multilead electrode: i) Electrocortical arousal was elicited from the thalamus, where the damage produced by the electrode was the same as in the caudate. ii) The absence of electrocortical arousal was confirmed when a unipolar needle electrode was used to stimulate the caudate. This electrode inflicted a minimum of damage due to the small volume of its pointed tip. Stimulation of the reticular nucleus of the thalamus through this electrode elicited diffuse, bilateral electrocortical arousal (Fig. 3 A). Stimulation in the center of the caudate at the same strength and at a strength up to three times the threshold found in the thalamus did not evoke electrocortical arousal (Fig. 3 B).

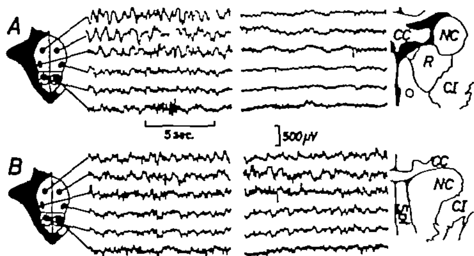


Fig. 3. Absence of electrocortical arousal (B) during and after stimulation of the caudate through unipolar electrode

Electrocortical arousal (A) with the electrode situated in the left reticular nucleus f the thalamus. Cat, encéphale isolé. Extradural, unipolar recording Recording sites shown to the left, stimulation sites indicated by black spots in the drawings to the right. The interruption in the middle of the records indicates an interval of 15 sec. (10 sec of stimulation with 1 msec pulses at 150/sec and 5 sec for recovery of the overloaded amplifier) Stimulus intensity 1.3 times threshold.

CC Corpus callosum. CI Capsula interna. NC Nucleus caudatus R. Nucleus reticularis thalami. Spc. Area septalis.

It has been reported that electrocortical arousal can be elicited from the caudate even in animals anesthetized with barbiturates (SHIMAMOTO and VERZEANO 1954) In the experiments reported here, the low voltage fast ac tivity produced after timulation of the thalamus and the part of th caudate adjoining it could no longer be elicited immediately after the intra enous injection of small doses of nembutal (7.5 mg/kg)

Discussion

The ascending reticular ctivating system (MORUZZI and MAGOUN 1949) can be defined as the subcortical regions from which electrocortical arousal can be induced by electrical stimulation excluding the classical sensory systems. The findings presented in this study show that the caudate nucleus is not a part of this system. This is in agreement w th th results of STARZL *et al* (1951) but contrary to the results of ther uthors (GERSHTZOFF 1941 SHIMAMOTO and VERZEANO 1954 STOUPEL nd TERZUOLO (1954) and inconsistent with the view recently expressed by JUNG nd HASSLER (1960 p 915) that the higher extrapyramidal centers and the nonspecific tivation system" are closely

related. Failure to find electrocortical arousal during stimulation of the caudate nucleus extends previous findings (LAURSEN 1961) that the caudate has no influence on cortical electrical activity

The electrocortical arousal response ascribed to stimulation of the head of the caudate nucleus was thought to be mediated by a subcortical relay (STOUPEL and TERZUOLO 1954) more specifically the intralaminar thalamic nuclei, because lesions here abolished the response (SHIMAMOTO and VERZEANO 1954) The alternative explanation emerging from the findings presented here is, that it was in fact spread of stimulating current from the caudate to the thalamus which elicited the electrocortical arousal response.

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Acta physiol. scand. 1961. 53. 239—246

From the Geriatric Department, Södersjukhuset, Stockholm, Sweden

Heparin and Thrombocytopenia in Experimental Burn Injuries

By

STIG-ARNE JOHANSSON

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Abstract

JOHANSSON, S. A. *Heparin and thrombocytopenia in experimental burn injuries.* Acta physiol. scand. 1961. 53. 239—246. — In experimental burns a decrease in the number of circulating platelets with an ensuing decrease of 5-hydroxytryptamine (5-HT) in whole blood was found. In lung tissues an increase of 5-HT was detected. Pretreatment of the animals with heparin prevented these effects. Coagulation time measurements showed a prolongation of the coagulation time. An increase of the anti-thrombic substances in blood was found. It is suggested that heparin, being a strongly acid polyelectrolyte, acts on the platelets and their ability to absorb and release 5-HT and *prevents the onset of a transient intravascular* coagulation during burn injuries.

Burn injuries in rabbits are accompanied by a decrease in the number of circulating platelets with an ensuing release of 5-hydroxytryptamine (5-HT serotonin) (JOHANSSON 1960 a). In anaphylactic reactions the decrease in platelet count is prevented if heparin is given prophylactically before the shock dose (JOHANSSON 1960 b). Survival times of burned dogs treated with heparin is prolonged in comparison to those of untreated dogs (ELROD et al. 1951, HALLBERG and PITZELE 1959). As to the mechanism of the protective action of heparin in burn injuries nothing is known.

The purpose of the present investigation is to study whether heparin prevents the decrease in platelet count and inhibits the release of 5-HT in burn injuries.

Methods

Rabbits of both sexes weighing 2.5—3.0 kg were used in these experiments. The animals (including controls) were anesthetized with nembutal (20 mg/kg). About 20 per cent deep third degree burns were produced with hot water (65° C) in 2 min on 20 shaved animals. Ten animals received 50 mg heparin Vitrum containing 102 I. u. per

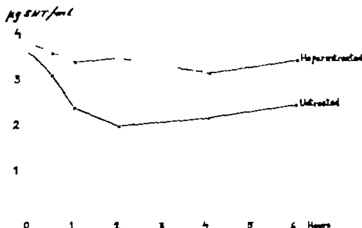


Fig. 1 The mean concentrations of 5-HT (µg 5-HT/ml) in whole blood from 5 untreated and 5 heparin treated animals at intervals after the burn injury Untreated animals ______ Heparin treated animals

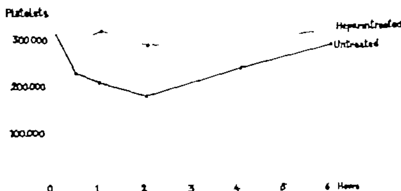


Fig. 2 Average platelet counts in whole blood from 5 untreated and 5 heparin treated animals at intervals after the burn injury Untreated animals ______ Heparin treated animals

mg 20 min before the burn injury Five untreated and five heparin treated animals were killed 90 min after the burn injury and intestines and lungs were homogenized with 0 1 n HCl and their content of 5-HT was determined after extraction into n-butanol. From the remaining ten animals samples of blood were withdrawn through polyethylene catheter in the carotid artery one hour before and at 30, 60, 120, 240, 360 min after the burn injury Nine volumes of blood were collected into centrifuge tubes containing 1 volume of 3.8 per cent trisodium citrat ($Na_3 C_6 H_5 O_7 \cdot 2H_2O$). International Co lusteroid centrifuge tubes were used. All glassware was silicone treated.

Coagulation time measurements were made according to HEDENIUS (1936) Platelet counts were made by modified method of KRISTENSON (1937) The anti-thrombic substances were determined according to BLOMBÄCK, BLOMBÄCK and WALLÉN (1955).

The 5-HT concentrations in whole blood and tissues were determined spectrophotofluormetrically after extraction into purified n-butanol according to UDENFRIEND, WEISSBACH and CLARK (1955) and WEISSBACH, WAALKES and UDENFRIEND (1958)

Table I. Platelet counts in thousands per cu. mm before and at intervals after the experimental burn injury

Untreated animals	Before the burn injury	Minutes after the burn injury				
		30	60	120	240	360
Rabbit 1	325	235	250	230	170	—
Rabbit 2	320	150	160	80	—	—
Rabbit 3	315	285	280	273	325	340
Rabbit 4	305	270	255	205	220	245
Rabbit 5	300	190	195	140	—	—
Heparin treated animals	**Before the burn injury**	**Minutes after the burn injury**				
		30	60	120	240	360
Rabbit 6	360	310	320	300	310	325
Rabbit 7	340	315	330	315	315	320
Rabbit 8	340	300	320	305	300	300
Rabbit 9	310	300	315	290	305	305
Rabbit 10	300	280	300	265	255	280

Mean value with standard deviation of 50 platelet counts from 5 rabbits anesthetised with nembutal 310 ± 15.

Results

The concentrations of 5-HT and the platelet counts in rabbit blood before and at intervals after the burn injury are shown in Fig. 1 and 2. In experimental burns there occurs a decrease in the total amount of 5-HT in rabbit blood due to a diminution in the number of circulating platelets (Table I). The platelets however return to normal levels within 6 hours, but the decrease in the 5-HT content of blood persisted for at least 12 hours. In platelet deficient plasma, however, no detectable amount of 5-HT was detected.

In those animals which had been pretreated with heparin the burn injury was accompanied by no decrease or only a slight decrease in the number of circulating platelets and in the 5-HT content of whole blood (Fig. 1—2, Table I).

The amount of 5-HT in intestines was high and because of the wide normal variation a release of the amine from the intestines would be difficult to detect. Analyses from 5 animals one hour after the burn injury gave values within the normal range. An elevation of the 5-HT level in the lung was observed 90 min after the burn injury (Table II). In 5 burned animals treated with heparin no significant increase in the 5-HT content was detected. Three of the unheparinized rabbits died within 6 hours while none of the heparinized animals died within this period (Table III).

Table II Amount of 5-HT in intestines, lung and whole blood 90 min after the burn injury in control rabbits from controls. (Range used where the analyses differed by more than 1 µg/g wet tissue. In other cases mean and standard deviations are shown.) (5-HT µg/g wet tissue)

	Controls	90 min after the burn injury	
		Untreated	*Heparin treated*
Number of animals	5	5	5
Intestine	8–12	8–13	8–14
Lung	2.4 ± 0.3	3.8 ± 0.3	2.6 ± 0.2
Whole blood	3.6	2.3	3.8

Table III The survival time of heparin treated and untreated animals after experimental burn injuries

	Number of animals	Animals alive after 6 hours	Animals alive after 10 hours
Untreated	5	2	1
Heparin treated	5	5	4

Table IV The anti-thrombic titre in seconds before and at intervals after the burn injury from 5 untreated animals

	Before the burn injury	Minutes after the burn injury 30	60	120	240	300	The animal died after
Rabbit 1	40	142	208	223	92	–	320 min.
Rabbit 2	56	92	102	119	–	–	150 min.
Rabbit 3	56	160	254	265	120	81	Alive after ten hours
Rabbit 4	59	86	93	120	102	66	420 min.
Rabbit 5	57	110	180	116	–	–	220 min.

Table V The coagulation time before and at intervals after the burn injury

Rabbit	Before the burn injury	Minutes after the burn injury 30	60	120	240	300
1	3′50″	8′20″	12′20″	14′10″	> 30	–
2	4′15	7′35	14′15	16′30″	–	–
3	3′30″	6′25	12′00	11′25″	10′45	6′05
4	3′45	6′50″	7′10″	14′30″	> 30	> 30
5	4′25	10′15	14′50″	16′15″	–	–

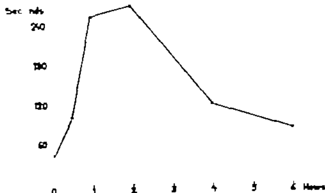


Fig. 3. The anti-thrombic titre in seconds at intervals after the burn injury in one of the animals.

To study whether the animals release heparin to the blood after the burn injury the anti-thrombin titre was determined in 5 unheparinized animals. The results are given in Table IV This study shows that an anti-thrombic substance, probably heparin, is released in large amounts in the first hours after the burn injury The activity of the anti-thrombic substance corresponds to between one and two i. u. heparin per ml whole blood. The coagulation time measurements from unheparinized animals are shown in Table V

Within 30 min of the burn injury there was a prolongation of the coagulation time and this prolongation persisted in the four animals which died within 7 hours of the burn injury

Discussion

An increasing amount of interest has been displayed in recent in the role of platelets in blood clotting and hemostasis. They supply agents active in both onset and in other phases of the clotting process. They also have the ability to form white thrombi (VAN CREVELD 1954) Finally they contain a vasoconstrictor substance, 5-HT which is liberated in the coagulation of blood where the platelets are disrupted (ZUCKER and BORRELLI 1954) Almost all the 5-HT circulating in the blood is bound to the platelets (HUMPHREY and JAQUES 1954) Deep venous thrombosis and pulmonary embolism are rather common complications of burn injuries (ALLGÖWER and SIEGRIST 1959) A decrease in platelet number has been noted in man as early as a few hours after the burn accident (MAC DONALD et al. 1945) and this study confirms this finding

After burning there occurs delayed eosinophilia, which might result from sensitisation following the absorption of altered proteins in the burned area (SEWITT 1951) FEDOROV (1956 and 1959) holds that the body is flooded with antigen derived from the burned skin and that this is responsible for part of the burn syndrome as a result of an autoimmunisation reaction.

In rabbits there is a marked diminution in the circulating number of platelets a few hours after the burn injury accompanied by a decrease in the whole blood concentration of 5-HT. Because of the rapid metabolism of liberated 5-HT (JOHANSSON 1960 b) a decrease in the number of circulating platelets thus produces a corresponding drop in the 5-HT content of the blood.

To study whether 5-HT is released from other sources than platelets the content of the amin in intestines and lung tissues was determined. No change in the content of 5-HT in intestines could be detected. An increase in the 5-HT concentration in lung tissues was however found probably due to increased amounts of platelets in this tissue.

AVDAKOFF in 1876 reported that blood of burned animals was toxic to other animals. Toxic substance from the burned skin or the blood are alleged to produce illness and even death. The experimental findings and the number and variety of substances proposed are voluminous but conflicting. Nevertheless various substances are undoubtedly released or newly formed after burn injuries (SEWITT 1957, ALLGÖWER and SIEGRIST 1957). Experimentally it is important to establish whether in-vivo inhibition of the formation or the release of such substances can reduce the oedema and be useful in the treatment of burn injuries.

Burn injuries are nearly always followed by oedema, although little is known about its exact mode of formation. It is interesting to note, however, that subcutaneous administration of 5-HT causes a marked increase in capillary permeability (ROWLEY and BENDITT 1956). The local oedema producing effect of subcutaneous injections of protein or dextran in rats is inhibited if the animals have been pretreated with the 5-HT liberating substance, reserpine (WEST 1957). 5-HT also increases the permeability of the beet root cell membrane to water-soluble pigments (PICCLES and SUTCLIFFE 1955). Along similar lines PICCLES (1956) found that pretreatment of erythrocytes with 5-HT accelerates their subsequent hypotonic hemolysis. 5-HT thus seems to produce alterations in the permeability of the cell membrane.

The released amounts of 5-HT in rabbit blood after experimental burn injuries are too small to cause death if injected intravenously into rabbits but many of the contributing symptoms which appear might be produced by 5-HT.

ELROD, MC CLEERY and BALL (1951) found that the survival times of burned dogs treated with heparin was twice that of untreated animals. They suggested that the effect was due to improved renal function and resorption of oedema fluid.

The present study shows that an anti-thrombic substance, probably heparin, is released in blood after burn injuries, and that this may be the cause of the prolonged coagulation time measurements. In two rabbits, however the coagulation time exceeded 30 min, which is too long a time to be caused by the released amount of anti-thrombic substance. It is interesting to note that the rabbit with

the highest platelet count throughout, had the highest anti-thrombic titre and was the only untreated animal to survive.

As to the mechanism of the protective action of heparin in burn injuries nothing is known. In an earlier study it has been shown that heparin has no or very little ability to combine with 5-HT at a pH and a salt concentration corresponding to that in blood (JOHANSSON 1960 b) Heparin thus probably does not act in blood by binding released 5-HT Thrombocytopenic blood can be kept fluid for hours by the addition of a very small amount of heparin (ALLEN et al. 1947) Whether the platelets absorb heparin on their surface or not is not known. Heparin, however produces a marked increase in the negative charge of platelets in citrate buffer (ROSS and EBERT 1959) The anti-thrombic effect of heparin is well known. A possible role of heparin in burn injuries would therefore be to prevent the onset of intravascular coagulation. This study shows that large amounts of an anti-thrombic substance, probably heparin, is released into rabbit plasma during burn injuries. Preliminary results from other investigations have shown that protrombin proconvertin and fibrinogen are decreased after the burn injury (JOHANSSON 1961) Transient intravascular coagulation after burn injuries might then be responsible for part of the appearing shock symptoms and death in burn injuries. Heparin thus may have a protective action in burn injuries by its direct action on platelets and its influence on different humoral coagulation factors.

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From the Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia 4, Pa., U. S. A.

Modifications of the Febrile Response to Pyrogen by Hypothalamic Heating and Cooling in the Unanesthetized Dog

By

H. T. Andersen, H. T. Hammel and J. D. Hardy

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Abstract

Andersen, H. T., H. T. Hammel and J. D. Hardy. *Modifications of the febrile response to pyrogen by hypothalamic heating and cooling in the unanesthetized dog.* Acta physiol. scand. 1961. 53. 247—254. — Experiments have been carried out with the aim of obtaining additional information on the mode and site of action of pyrogens. On the assumption that these agents act on the thermally sensitive cells of the anterior hypothalamus by raising the "set point" for physiological temperature regulation to a higher level, it was planned to counteract their effect by heating this region of the hypothalamus with 3.7 megacycle radio frequency energy between implanted thermodes, or enhance their effect by cooling the same area by circulating water through the thermodes. Temperatures were measured by means of thermocouples inserted into the thermodes. The rectal and the skin temperatures were also recorded by means of thermocouples. Fever was induced in the animals by intravenous administration of a pyrogenic polysaccharide obtained from Pseudomonas. The body temperature started to increase 30—45 minutes after the injection and usually remained at the elevated level for 3 hours or more. Heating of the hypothalamus was performed 1) immediately after administration of pyrogen, 2) while the fever developed and 3) when the fever condition was maximal. In the first case the fever failed to develop, in the second instance the rise in rectal temperature was stopped, and in the third event, thermoregulatory responses similar to those obtained with the same technique at normal body temperature were elicited. In the experiments in which the pyrogenic action was enhanced by hypothalamic cooling "hyperfever" was produced.

Present address: Department of Physiology, Kungl. Veterinärhögskolan, Stockholm 51, Sweden.

The site and mode of action of bacterial pyrogens remain a question of considerable controversy although much work on the problem has been performed by several workers.

Fevers induced by the administration of pyrogenic substances involve both an inhibition of the mechanisms subserving heat loss, and a stimulation of those regulating heat production (PARK and PALMES 1948, THOMPSON 1959).

LIEBERMEISTER (1875) suggested that fever is caused by an upward displacement of the "set point" temperature at which the centers controlling body temperature regulate. This resetting hypothesis was adopted and developed further by DU BOIS (1941).

CHAMBERS *et al.* (1949) studied the febrile response to bacterial pyrogens in normal cats and dogs, as well as in preparations with different lesions in the central nervous system. They found that decortication, and thalamic and caudal hypothalamic lesions did not prevent the febrile response, whereas decerebrate preparations with most of the midbrain intact failed to show such an effect. GRANT (1949) found no evidence that pyrogenic substances influence the hypothalamic temperature regulating centers. He suggested that their action is one of interference with motor mechanisms of the lower levels of the brain stem.

HALL *et al.* (1951) stated plainly that the action of bacterial pyrogens is not a resetting of the primary thermoregulatory centers of the hypothalamus, but that the thermostatic disturbances observed are elicited from a general interference of these substances on the autonomic functions controlled by the brain stem. THOMPSON (1959) on the other hand, showed very convincingly that dogs in which the grey matter of the posterior hypothalamus had been completely extirpated, were unable to develop a response to bacterial pyrogens.

Further information on the site of action of the bacterial pyrogens would probably be derived if their action could be counteracted or enhanced by respectively heating or cooling the thermosensitive cells of the hypothalamus during various phases of the developing fever. The results of such experiments are reported in this paper.

Material and Methods

Four mongrel dogs were used for the experiments. Fever was induced by intravenous administration of "Piromen"[1].

Because dogs develop a refractoriness towards the pyrogenic action, only 2—3 exp. were performed on each individual with 2—4 weeks interval between runs. The initial dose was 0.6 μg/kg body weight. Subsequent doses were increased by 0.2 μg/kg body weight in each additional experiment. These doses invariably caused a fever of roughly 1° C, and because of the moderate dosage, the side-effects, retching, vomiting and defecation were only observed in 2 exp. The pyrogenic action of the bacterial polysaccharide was counteracted by heating the anterior hypothalamus with 3.7 megacycle radio frequency energy and enhanced by cooling of the same area with circulating

[1] Pseudomonas polysaccharide manufactured by Travenol Laboratories, Inc., Morton Grove, Ill., U. S. A.

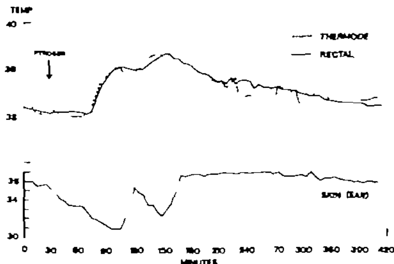


Fig 1 Normal development of pyrogenic fever The usual two-step rise in rectal and hypothalamic temperature is shown. Note cutaneous (ear) vasodilatation between steps.

water. The rectal temperature was used to indicate the febrile response, and the ear temperature was measured for an indication of vasomotor activity In the experiments in which the hypothalamus was heated the temperature of the thermodes were recorded, and *in the experiments where cooling was employed both the stimulating and the* hypothalamic temperatures were similarly measured. In these latter experiments an average skin temperature was also recorded. The details of the techniques employed have been described elsewhere (HAMMEL *et al.* 1960, ANDERSSON *et al.* 1960)

The following types of experiments were carried out

A. Heating of the hypothalamus 1) immediately after administration of pyrogen, 2) while the fever developed and 3) when the fever condition was maximal.

B. Cooling of the hypothalamus during the development of pyrogenic fevers so that the temperature of the hypothalamus was kept well below the rising rectal temperature.

All experiments were carried out t environmental temperatures of 27—28° C.

Results

Normal development of pyrogenic fever

A typical development of the febrile esponse to I. v administration of Piromen is shown in Fig 1 The thermode temperature is, in this experiment, the temperature of the anterior hypothalamus. The familiar two-step development of the pyrogenic fever which has been noticed by several investigators (CHAMBERS *et al.* 1949 THOMPSON 1959) is clearly seen. The period of time which elapsed from the administration of pyrogen until the rectal and hypothalamic temperatures started rising was 35 to 45 min. The ear temperature mostly fell during this "latency period One very interesting feature of this curve is that in the normal development f pyrogenic fever the hypothalamic temperature was always higher than the rectal, whereas during the first hour of defervescence the hypothalamic temperature was the lower of the two. In

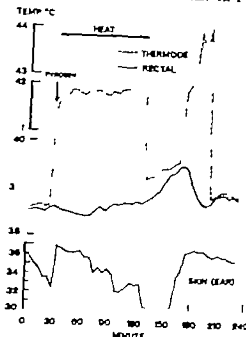


Fig. 2. Inhibition of the febrile response to Piromen by heating the stimulating thermodes to 41.8° C after the administration of the pyrogen. The normal response followed immediately after discontinuing the thermal stimulus.

the late stages of defervescence the hypothalamic temperature commonly started fluctuating around the rectal. Shivering was invariably exhibited during the fever development, and panting likewise during defervescence.

Hypothalamic heating immediately after administration of pyrogens

In order to inhibit the development of fever after the administration of pyrogen the anterior hypothalamus was heated as soon as Piromen had been injected. The result is presented in Fig. 2. The ear temperature rose in response to the heating, and the rectal temperature fell slowly. After a normal latency of 45 min a period followed during which the ear temperature fell throughout. The animal exhibited a small rise in the rectal temperature, but only enough to bring it up to 0.2° C above that of the period before pyrogen was administered. During the continued heating of the hypothalamus this level of rectal temperature was maintained without significant changes for 45 min. When the heat stimulation of the hypothalamus was cut off the ear temperature fell abruptly. The rectal temperature rose rapidly 1.0° C above the resting level. By heating the anterior hypothalamus to a higher temperature than before it was possible to induce cutaneous vasodilation as seen from the ear temperature, and a precipitous fall in the rectal temperature. When the heating was stopped, the rectal temperature increased by 0.5° C and then levelled off.

Inhibition of a developing fever by hypothalamic heating

Another experiment is shown in Fig. 3. Pyrogen was given, and the thermode temperature was kept only 1.5° C above the rectal temperature. Due to this

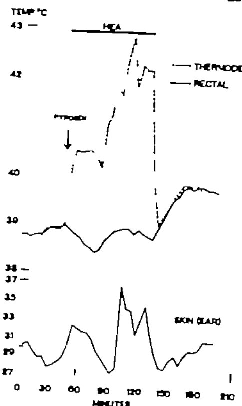


Fig. 3. Inhibition of pyrogenic fever during its development due to hypothalamic heating. The febrile response was obtained upon discontinuation of the thermal stimulus.

slight stimulation, the ear temperature indicated vasodilation, and the rectal temperature fell roughly 0.6° C. Thirty min after the injection the ear temperature started decreasing the fever developed accompanied by shivering, and the hypothalamic temperature fell in spite of unchanged stimulation. The rectal temperature increased rapidly By increasing the stimulating temperature of the thermode to 42—43° C it was possible to inhibit the developing fever and lower the rectal temperature due to vasodilation (see ear temperature) and panting Stoppage of the heat supply to the hypothalamus brought about vasoconstriction, vigorous shivering and an brupt rise in rectal temperature to 1.2° C above the resting

Effect of hypothalamic heating on fully developed pyrogenic fever

Fig 4 shows an experiment n which th fever was allowed to develop normally When a stage had been reached at which the rectal temperature levelled off after having increased 1.0 C above normal, the anterior hypothalamus was heated as shown. Th responses elicited were strong cutaneous vasodilation and marked fall in rectal temperature of 0 6 C. Removal of the stimulus caused vasoconstriction, shivering and a second rise in rectal temperature almost to the sam level as the first. The sam sequence of events followed

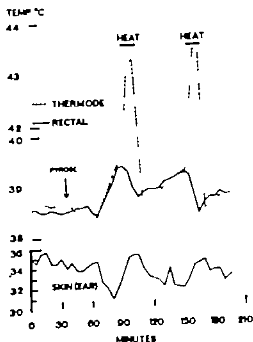


Fig 4 Hypothalamic heating applied in the case of fully developed pyrogenic fever The thermal responses elicited were very similar to those obtained at normal body temperature.

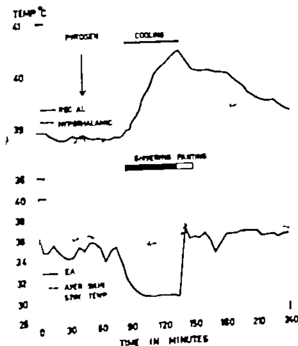


Fig. 5. The additive effect of injected pyrogen and hypothalamic cooling A "hyperfever" was produced.

repeated stimulation, and a third rise in rectal temperature was produced after the heating had been discontinued. This shows that the pyrogenic substance was exerting its effect throughout the experiment.

Hypothalamic cooling and pyrogenic fever development

In the experiments in which the anterior hypothalamus was cooled after the pyrogen had been given, a summation of these two stresses was obtained. The result, an elevation of the rectal temperature well above the level corresponding to the pyrogen alone, is demonstrated in Fig 5 As soon as the cooling was stopped, the ear temperature increased 7° C, and the animal started panting The average skin temperature also rose conspicuously This situation, however lasted for only 10—12 min, during which the rectal temperature fell from 40 4 C to 40 0° C. The rectal temperature levelled off at this point and remained almost constant for 35—40 min before the onset of defervescence. The hypothalamic temperature also rose during the stages in which the fever developed. This was inevitable in order that the temperature of the circulating water should be held within relatively physiological limits (38—33 C) An experiment was performed where the hypothalamic temperature was kept almost constant, but to obtain this constancy the stimulating temperature had to be lowered to 26.8° C. The findings in this latter experiment were essentially the same as those reported in Fig 5 except that a peak of 41 1 C in rectal temperature was reached. After abolishing the cold stimulation, the rectal temperature due to the pyrogen alone was found to be 40.6° C.

Discussion

The negative or positive summation of an injected pyrogenic substance and hypothalamic heating or cooling respectively during the febrile response has been demonstrated by the experiments reported in this paper The findings that it is possible to suppress the fever from developing by heating the thermosensitive structures in the anterior hypothalamus, and likewise to enhance the response to the pyrogen by local cooling of this same area, may be interpreted as indicative of the correctness of the thermostatic resetting theory Especially because the thermal stimuli employed have been moderate, it seems probable that the pyrogen dministered acts on the physiological thermostat directly Hence, when this area is locally heated, the induced temperature change satisfies the requirements of the thermostat and prevents general fever development. Furthermore, the hyperfever produced by moderate, local cooling shows the additive effect f two stresses working in the same direction, and it is quite conceivable that both timuli are exerting their effect through the thermosensitive structures in the anterior hypothalamus. There can be littl d ubt, however that Piromen" and probably other pyrogens, act at other locations in the central nervous system, as evidenced by the side-reactions, retching, vomiting and defecation which may be observed when larger doses of pyrogens are given.

At the present time one must consider two alternative explanations of our findings in addition to the one outlined above.

1. The pyrogenic substances act on other structures in the central nervous system in addition to the thermoregulatory cells in the anterior hypothalamus. Displacement of the temperature of the latter will, however, respectively counteract or enhance the effect exerted on these structures as described.

2. The pyrogenic substances act exclusively on central nervous structures other than physiological thermostat, but in such a way that the result is a general rise in body temperature. In this case one is, in our experiments, simply left with two different physiological stimuli which have different sites and modes of action, but which will summate algebraically in their thermal effect.

It seems, however, that these two alternative theories are rendered less likely by the results reported in the present study because the stimulating temperatures employed were about the same magnitude as used to produce the same thermoregulatory effects in the normal dog. Especially if alternate theory no. 2 is correct, one would expect that the amount of hypothalamic heating needed to counter the fever drive from extra-hypothalamic origin would be larger than it was actually found to be.

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From the Department of Pharmacology Karolinska Institutet, Stockholm, Sweden

Augmentation of Cardiac Contractile Force and Heart Rate by Medulla Oblongata Stimulation in the Cat

By

Anders Rosén

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Abstract

Rosén A. *Augmentation of cardiac contractile force and heart rate by medulla oblongata stimulation in the cat.* Acta physiol. scand. 1961 53. 255—269. — Electrical stimulation in two relatively well circumscribed regions of the medulla oblongata elicited tachycardia and augmentation of the right ventricular contractile force recorded by strain gauge arch technique. One of the two regions was found to be situated within the more central part of the pressor area. Stimulation in this region generally produced vasoconstriction in skeletal muscle parallel with the increased cardiac activity. The second region was found to be located in the "sympathetic vasodilator area" in the ventrolateral part of the medulla. Muscle vasodilatation elicited by stimulation in that region was accompanied by an increase in the cardiac functions. The augmentation of cardiac activity with stimulation of the two regions was mediated by the sympathetic innervation of the heart and, to minor extent, by an elevated secretion from the adrenal medulla.

A tachycardia following stimulation in the medulla oblongata has been reported to occur by e.g. Chen et al (1936), Mossner (1939), Mc Queen et al (1934), Amoroso, Bell and Rosenberg (1954) and Peiss (1960). However the literature contains very few data on augmentation of the cardiac contractile force with such stimulation. Peiss (1958) observed, on stimulation in the pressor area, alterations of pulse pressure which were interpreted as signs of an increased force of myocardial contraction. The finding of Randall and Rohse (1956) that stimulation of the cardiac sympathetic nerves increases the force of

cardiac contraction without necessarily raising the heart rate suggests that separate fibers exist for these two functions. While the hypothalamic representation of the inotropic fibers to the heart has been subjected to relatively thorough study (RUSHMER, SMITH and FRANKLIN 1959 SMITH *et al.* 1960, ROSÉN 1961 a) their representation in the medulla oblongata has been treated only in the above mentioned investigation of PEISS.

The present investigation was designed to establish whether the sympathetic vasodilator outflow which passes through the ventrolateral portion of the medulla (LINDGREN and UVNÄS 1953 LINDGREN *et al.* 1956) is accompanied by fibers the stimulation of which produces cardiac inotropic and chronotropic effects. Electrical stimulation of the vasodilator pathway in the central nervous system elicits, in the cat and the dog a cholinergic sympathetic skeletal muscle vasodilatation. Parallel with the vasodilator response, increased liberation of catechol amines from the adrenals occurs (GRANT *et al.* 1958, LINDGREN, ROSÉN and UVNÄS 1959a) Hypothalamic stimulation of the vasodilator system causes, in addition, excitation of inotropic and chronotropic fibers to the heart (ROSÉN 1961a) The question arose whether the concomitance of muscle vasodilator and of cardiac responses on stimulation in the hypothalamus was purely coincidental or whether it signified an interrelationship. Should the vasodilator outflow prove to be accompanied by cardio-active fibers even at other levels of its central course, the possibility of an interrelationship would be strengthened. The vasodilator outflow in the medulla oblongata is well suited for such complementary studies, for in that part of the brain stem its course is relatively well separated from those of other vasomotor regulating structures — The pressor area will also be studied with respect to the occurrence of cardio-active structures, since it would be of interest to verify by direct recording of the myocardial contractile force, Peiss's finding — in studies of blood-pressure variations — that an increased cardiac contractile force frequently accompanies stimulation in the pressor area.

Method

Twenty-five successful experiments were performed. The cats, weighing between 1.7 and 3.7 kg, were anesthetized with chloralose (25—50 mg/kg) and urethane (200—500 mg/kg) The rectal temperature was maintained at 37° C with the aid of an infrared lamp directed towards th abdomen and thorax.

The arterial pressure was measured with Statham transducer (P 23 A) via a plastic cannula inserted into a carotid artery and advanced so that its tip was situated in the aortic arch. The muscle blood flow was recorded in the right femoral artery of a skinned leg In order to exclude blood flow from the paw a tight ligature was placed just above the ankle. The blood flow in the femoral artery was conducted, via a plastic cannula, to a silicone-filled drop chamber (LINDGREN 1958) where the number of drops was registered by an ordinate recorder via a photocell. For the maintenance of warmth and moistness the detached skin was replaced around the muscle. To prevent coagulation, heparin (5 per cent, Vitrum) was administered intravenously in a dose of 25 mg/kg Dextran (Macrodex, Pharmacia) was given intravenously in doses sufficient to com-

pensate blood losses. Positive-pressure artificial respiration was maintained during the experiments. In 12 animals bilateral cervical vagotomy was done before the experiment, and in 8 of these that carotid artery which had not been cannulated for blood-pressure measurement was occluded prior to bulbar stimulation and remained occluded throughout the experiment, in order to eliminate any cardiac effects which might have arisen via baroceptor mechanisms.

The Horsley-Clarke technique was used for stimulation. The skull was perforated with dental drill so that an electrode could be introduced in the medulla on the right side. Both unipolar (6 experiments) and bipolar (19 experiments) electrodes were employed. The unipolar electrode was 0.45 mm in diameter and had a pointed, uninsulated tip 0.3 mm long. The bipolar electrode consisted essentially of two unipolar electrodes the uninsulated tips of which had been so ground as to form flat oblique surfaces, one reaching 0.5—0.7 mm deeper than the other (conforming in principle to the illustration under B, Fig. 2, page 36, LINDGREN 1955). Electrical stimulation, 1.5—2.5 V, 70 cps, pulse duration 2 msec, was produced by square-wave generator.

The two stellate ganglia were carefully exposed via an approach through the thoracic wall between the first and second rib on each side. The thoracic wall was thereupon closed. It was later reopened for removal of the ganglia, then closed again. The adrenals were mobilized via an abdominal wall incision for later ligation and removal when required.

The right intraventricular pressure was recorded, in 14 experiments, by means of Statham transducer (P 23 A). Following thoracotomy polyethylene catheter was advanced through the ventricular wall int the cavity with the aid of pointed metal guide which was then removed. The heart rate was measured with an interval recorder (GOLDSCHMIDT and LINDGREN 1961) via impulses from the blood-pressure channel of Grass (Polygraph) instrument.

The myocardial contractile force was measured with a strain gauge arch. Following thoracotomy and incision of the pericardium the strain gauge arch was attached, in all experiments, to the right ventricular wall by means of sutures which penetrated deeply in the heart muscle. The sutures were so placed as to avoid traumatization of any major coronary vessel. The muscle segment between the two points of attachment was conventionally stretched 40—50 per cent, as described by COTTEN and BAY (1956) in order to minimize those disturbances in recording which attend enlargement of the ventricle. The thoracic wall was subsequently closed as far as possible for the maintenance of warmth and moistness. Throughout the recording the intrathoracic pressure was atmospheric.

In 7 experiments use was made of an open strain gauge arch similar in essentials to that described by BONIFACE, BRODIE and WALTON (1953). For the remaining 18 experiments somewhat modified, and far more sensitive arch (ROSÉN 1961 a) was employed. For further details of and comment on the strain gauge arch technique in measurement of cardiac contractile force, reference should be made to previous investigations (COTTEN 1953, COTTEN and BAY 1956, ROSÉN 1961).

Results

Cardiac Responses to Stimulation in the "Sympathetic Vasodilator Area"

The stimulation took place, in all experiments, within that area of the sympathetic vasodilator outflow which LINDGREN *et al.* (1956) observed in the ventrolateral portion of the medulla oblongata. In 9 experiments such stimulation elicited vasodilatation in the skeletal muscles. That the effect was a

Table I. Responses of cardiac contractile force (C. F.) and heart rate (H. R.) to bulbar stimulation of the sympathetic vasodilator pathway eliciting a muscle vasodilator response

	C. F	H. R.	After adrenalectomy C. F	After adrenalectomy H. R.	After stellectomy C. F	After stellectomy H. R.
Cat no. 1 A	+++	+				
» » 2 A	increase[1]	+				
» » 3 V	+	+++	+	+		
» » 4 V PCO	++	+	+	+		
» » 5	+++	+	+++	+		
» » 6 V	++	+++	++	+++	0	0
» 7	+++	++	++	+	0	0
» 8 V PCO	++	+			0	0
» 9	+	+			0	0

[1] adrenaline injected.

A = Adrenalectomized.
V = Vagotomized.
PCO = Persistent carotid occlusion.
C. F +++ correspond to the response of about 1.0 µg/kg adrenaline i.
++ » 0.5
+ 0.2
0 insignificant response.
H. R. +++ 15—20 % increase.
++ 10—15 %
+ 5—10 %
0 < 5 % (Insignificant response)

cholinergic vasodilator one attributable to the sympathetic vasodilator system was evident from the fact that on stimulation following atropinization it was either substantially reduced or absent. The activations invariably gave rise to an increase in the heart rate and in the cardiac contractile force concomitantly with the vasodilatation (Table I). One of the experiments is illustrated in Fig. 1 (cat 3 Table I). All effects, in this as well as in the remaining 8 experiments, occurred after a maximum latency of 4 sec (for the heart rate, 2 sec). Neither in this nor in other experiments were the cardiac responses affected by atropine. In 4 experiments the right ventricular pressure was recorded. The systolic pressure rose, in each instance, commensurately with the increase of contractile force; the diastolic pressure, on the other hand, remained unchanged.

The magnitude of the cardiac responses is evident from Table I. The augmentation of cardiac contractile force was equivalent to the elevation produced by adrenaline, 0.2—1.0 µg/kg, administered intravenously shortly before or after the stimulation. This method of estimating the responses of the cardiac contractile force was found to be appropriate for experiments of this type (Rosén 1961a). The rise in the heart rate on stimulation amounted to between 5 and

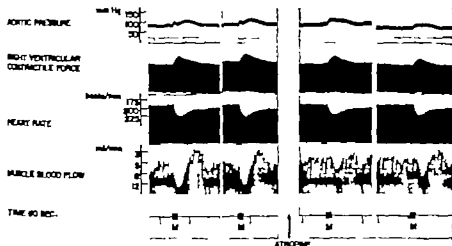


Fig 1. Cat 3. Aortic pressure, right ventricular contractile force, heart rate and muscle blood flow responses to stimulation in the entrolateral portion of the medulla oblongata before and after atropinization.
M. Stimulation 2.0 \ 70 cps.
Atropine 0.5 mg/kg intravenously
Note that there are less increases in muscle blood flow after atropine.

20 per cent. The cardiac responses were mediated generally by the sympathetic innervation to the heart. This finding was corroborated not only by the short latency but by bolition of responses by stellate ganglionectomy Th experiment illustrated in Fig 2 showing the cardiovascular responses to stimulation of the sympathetic vasodilator tract, is one of the four in which the stellate ganglia were removed (T ble I) In this experiment, as in the others, the augmentation of the cardiac responses was practically eliminated by the ganglionectomy In some experiments bilateral adrenalectomy reduced the cardiac responses to stimulation, but in others t did not. In the former instances catechol amines were evidently released from the adrenals in amounts sufficient to influence the cardia functions. The amounts liberated in these experiments did not, however suffice to affect ppreciably the vasomotor tonus in the muscles.

During the attempts to acti ate the sympathetic vasodilator pathway within its circumscribed area in the entrolateral portion of the medulla, ncreased cardiac activity was observed to accompany 10 stimulations which produced no significant muscl vasodilatation. In one instance however tropine had been administered earlier and in some experiments stimulation was associated with a minor increase of blood flow which may have resulted from activation of the vasodilator pathway F ve stimulation points were localized histologically all of them within a region coinciding w th the sympathetic vasodilator area"

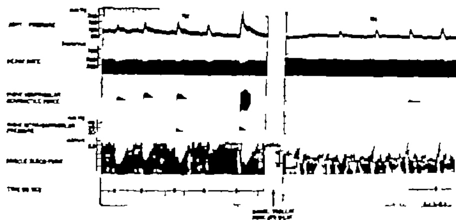


Fig 2. Cat 8. Aortic pressure, right ventricular contractile force, right intraventricular pressure, heart rate and muscle blood flow responses to stimulation at various levels in the oblongate medulla before and after atropinization and removal of the two stellate ganglia. The adrenals were intact.

1—5 Bulbar stimulations (2.5 V, 70 cps) delivered at five levels approximately 1 mm apart while the tip of the electrode was gradually shifted dorsally from the ventrolateral area.

Atropine 0.3 mg/kg intravenously

Note the minimal cardiac and also the muscle vasoconstrictor responses to stimulation 4, applied above "the sympathetic vasodilator area

(Fig 4) The cardiac responses were of the same type and magnitude and had the same latency as those induced by the stimulations which gave rise to vasodilatation (Table II) Adrenalectomy in the two experiments in which it was done, served to reduce the augmentation of cardiac contractile force (Table II)

Twelve of the experiments were conducted as follows. The electrode was first advanced through the dorsal part of the medulla to the most ventral portion of the ventrolateral area. Stimulation was then produced at different levels, generally separated by 0 5 to 1 mm, the electrode tip being withdrawn a little at a time. It was found that stimulation in a region above the vasodilator area in 8 exp. caused no significant changes either in the cardiac contractile force or in general, in the heart rate in 2 exp. it reduced the contractile force recording and gave rise to bradycardia concomitant with a substantial blood-pressure fall and in the remaining 2 exp it produced an augmentation of the contractile force. In 4 of those experiments in which no cardiac responses attended stimulation above the "vasodilator area" vasoconstrictor responses were observed in the muscles. One of these 4 exp. is illustrated in Fig 2 The first stimuli elicited vasodilator responses in the muscles, parallel with an augmentation of the cardiac contractile force and a minor tachycardia Stimulation no. 4 — with the tip of the electrode approximately 1 mm above that in no 5 — was associated with insignificant cardiac responses and a vasoconstrictor response in the muscles.

Table II Responses of cardiac contractile force (C. F) and heart rate (H. R.) to stimulation in the ventrolateral area of the oblongate medulla eliciting no muscle vasomotor response

	C. F	H. R.	After adrenalectomy C. F	After adrenalectomy H. R.
Cat no. 10 [1] PCO	+	+		
11	++	+		
12	++	++	+	++
13 [1] PCO	+++	+++		
14	++	+		
15	increase	+++		
16 A	+++	+++		
17	+++	not measured	++	
18	++	not measured		
19 V	+++	+		

No adrenaline injected.

[1] A, PCO, C. F (+++ ++ + 0) and H. R. (+++ ++ + 0) see table I.

Cardiac Responses to Stimulation in the More Central Portions of the Pressor Area

In 14 exp. stimulation was pplied in the more central parts of the region which ALEXANDER (1946) described as the pressor area. The elicited response consisted, in each instance, of an increased right ventricular contractile force, a heightened systolic pressure (diastolic pressure unchanged) in the right ventricle and, in general, tachycardia. In all except one of these experiments the stimulation was observed to be accompanied by varying degrees of muscle vasoconstriction. One of the experiments is illustrated in Fig 3 from which t will be seen that the stimulation elicited increased cardiac activity parallel with muscle vasoconstriction (the vasoconstrictor response was most conspicuous at the last stimulation) The augmentation of the cardiac responses was reduced following adrenalectomy Stimulation plus intravenous injection of adrenaline 1 μg/kg (M + A in the figure) produced effects on the heart similar in amplitude to those obtained prior to drenalectomy This suggests that catechol amines had been liberated from the adrenals to approximately the same extent, as the injected amount, and had influenced the cardiac functions. The rise in cardiac responses was nearly abolished following stellate ganglionectomy The adrenal catechol amines and the sympathetic innervation to the heart were thus both responsible, in this experiment, for the increased cardiac activity In another experiment with stimulation in the pressor area (stimulation no. 5 in Fig 2) the cardiac ugmentation mediated ia the cardiac sympathetic i nervation was predominant. The stimulation parameters were the same as those used for activation in the ventrolateral area, though pressor area stimulation gave rise to more

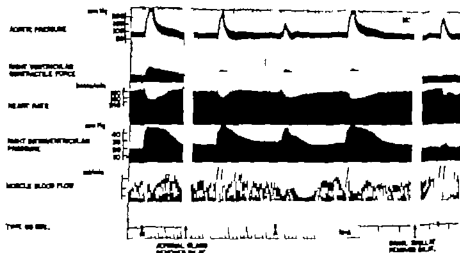


Fig. 3 Cat 21 Aortic pressure, right ventricular contractile force right intraventricular pressure, heart rate and muscle blood flow responses t stimulation in the pressor area before and after removal of both adrenals nd both stellat ganglia.
M. Stimulation 2.0 V, 70 cps
A. Adrenaline 1 0 μg/kg intravenously
Note that after adrenalectomy there is partial, and after stellatectomy an almost total, reduction of the cardiac responses to stimulation.

pronounced cardiac effects (compare Table I and II with Table III) Under the experimental conditions obtaining the augmentation of cardiac contractile force on stimulation in the pressor area was equivalent to the increase evoked by intravenous injection of adrenaline in a dose of 1 γ/kg or more (Table III) The rise in heart rate varied, in general, between 5 and 20 per cent in one instance it amounted to 50 per cent.

In 13 of the 14 experiments (the exception being cat 24 in Table III) the latency of the cardiac responses amounted to a maximum of 4 sec (for the heart rate 2 sec) This indicates that the responses were elicited, at least in part, via the sympathetic innervation to the heart. It will be seen from Table III that in the cases with short latency the cardiac responses were substantially reduced or altogether eliminated on stimulation applied following stellate ganglionectomy A reduction of cardiac contractile force resulted from ganglionectomy in itself, in general fairly small, though it occasionally amounted to almost 50 per cent. Parallel with that decrease n contractile force there was a minor fall of systolic pressure in the right ventricle as well as moderate bradycardia In one experiment (cat 24 Table III) approximately 10 seconds elapsed before the cardiac responses were observed. The same was true of cat 13 on stimulation following stellate ganglionectomy These observations suggest that humoral mechanisms were involved in the responses. In the other experiments too, such

Table III Responses of cardiac contractile force (C. F.) and heart rate (H. R.) to bulbar stimulation in the pressor region generally eliciting muscle vasoconstrictor response

	C. F	H. R.	After adrenalectomy		After stellatectomy	
			C. F	H. R.	C. F	H. R.
Cat no 9 V	+++	+++				
10 V PCO	++++	+++				
11	+++	+				
24 V PCO	+++	+	0	+		
25	+++	+	+	+		
20 V PCO	++++	+++	+++	++	0	0
6 V	+++	Increase 50 %	+++	++	0	+
21 V PCO	++++	+++	+++	++	0	+
22 V PCO	++++	++	++++	+	0	+
23 V	++++	++	++++	++	0	0
5	+++	0			0	+
8 V PCO	+++	++			0	0
12 A	+++	++			0	0
13 V PCO	+++	++			++	++

V A, PCO C. F (+++ ++ + 0) and H. R. (+++ ++ + 0) see table I
C. F ++++ correspond to the response of about 1.5 μg/kg or more of adrenaline i.

mechanisms — in addition to the nervous mechanism — may have played a role. In several experiments bilateral adrenalectomy was done prior to stellate ganglionectomy following which a reduction of cardiac responses was frequently observed (Table III) — a finding which suggested that catechol amines liberated from the adrenals on stimulation were a contributory cause of the increased cardiac activity It was not possible to determine, however whether the amount of catechol amines also sufficed to affect the vasomotor tonus in the muscles, since reliable evaluation was precluded by the very marked blood-flow and blood-pressure responses which were also present after adrenalectomy

Comment

Stimulation in the pressor area gave rise to such marked cardiovascular effects that the heart volume might have been changed. This factor is of particular significance in that a major degree of heart enlargement will affect the strain gauge arch recording With moderate enlargement, however the recording will not be appreciably influenced (COTTEN 1953, COTTEN and BAY 1956, ROSÉN 1961) In all of the present experiments in which both adrenalectomy and stellate ganglionectomy were done, and in some of those in which only one of the two operations was performed, subsequent stimulation caused no significant change in the recording of either the contractile force or the ventricular

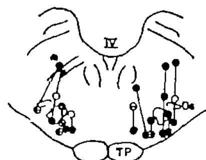


Fig. 4 Drawing of frontal section through the medulla oblongata of cat. Stimulation points, histologically localized to region 2.5 mm anterior or posterior to this section, are represented by the circles.
Os, Superior olive; TP Pyramidal tract IV Fourth ventricle.
◉ Cardiac augmentation and muscle vasoconstriction.
● Cardiac augmentation and muscle vasodilatation.
⊕ Cardiac augmentation and no muscle vasomotor response.
⊖ Blood-pressure fall, bradycardia and decrease in the recording of cardiac contractile force.
○ N cardiac response, but muscle vasoconstriction.
○ No cardiac response and no muscle vasomotor response.
The lines connect stimulation points referabl to one and the same electrode puncture.

pressure. It follows that any alteration of heart size on such stimulation was not sufficient to influence the recordings. The question remains as to whether stimulation with the adrenals and/or the stellate ganglia intact might have increased the heart volume sufficiently to affect the strain gauge arch recording As regards the adrenals, West and Rushmer (1957) found that in both unanesthetized and anesthetized dogs intravenous infusion of adrenaline or noradrenaline increased the left ventricular diastolic diameter Even with large doses of catechol amines the increase seemed, however to be relatively small. Both adrenaline and noradrenaline, even in small doses, augment the contractile force of the myocardium (e g West and Rushmer 1957 Rosén 1961b) There is reason therefore, to surmise that that part of the contractile force response which, in my experiments, disappeared following adrenalectomy was in fact attributable to a direct influence of adrenal catechol amines on the myocardial contractility As to the stellate ganglia, Arzola and Rushmer (1956) found in dogs that electrical stimulation of the left stellate ganglion in situ actually reduced the dimensions of both ventricles, presumably as a result of augmentation of the myocardial contractile force as well as acceleration of the heart rate. This suggests that on bulbar stimulation in my experiments intact stellate ganglia tended rather to counteract than to facilitate an increase in heart volume. Another observation which may indicate that the strain gauge arch recording was not appreciably influenced by volume changes, is that on bulbar stimulation the right ventricular systolic pressure almost invariably rose — parallel with the increase of contractile force — to not more than approximately double, while the diastolic pressure remained unchanged. Similar right ventricular pressure behavior had previously been observed in seven instances (Rosén 1961a one illustrated in his Fig 4) of experimentally induced enlargement of the right ventricle, with no significant effect on the strain gauge arch recording from the ventricle.

Histologic Localization

Fifteen cat brains were examined histologically After removal they were fixed in 10 per cent formaldehyde solution. Frozen serial sections were cut in a plane parallel to the electrode position. Twelve stimulation points were localized in the sympathetic vasodilator area" (LINDGREN and UVNÄS 1953 LINDGREN *et al.* 1956) 7 of them were associated with vasodilator responses and 5 with no vasomotor responses in the muscles (Fig 4) An additional 10 stimulation points were localized in the more central regions of the pressor area defined by ALEXANDER (1946) (6 of them are shown in Fig 4) 9 were associated with vasoconstrictor responses and one with no vasomotor responses in the muscles. Activation of each of these 22 stimulation points had increased the cardiac activity

Discussion

In the present investigation different regions in the medulla oblongata were stimulated and the resulting changes in the skeletal muscle vasomotor tonus and in the heart rate and cardiac contractile force were studied. It was found that both the myocardial contractile force and the heart rate were increased, pparently independently on bulbar stimulation in the more central portion of that region which ALEXANDER (1946) described as the pressor area. In general, stimulation at that site elicited — parallel with the cardiac responses — vasocon striction in the skeletal muscles. In several instances, moreover stimulation around the ventral border of the pressor area evoked vasoconstrictor responses in the muscles with no demonstrabl concurrent changes of cardiac functions. The results confirm PERSS (1958) claim that the myocardial contractile force may increase on stimulation in the pressor area, and that vasoconstriction elic ited by bulbar stimulation is not necessarily associated with cardiac responses. According to my observations the elevated cardiac contractile force arose mainly via excitation of sympathetic fibers to the heart. The fact that activation of the pressor area in my experiments was also attended by cardio-acceleration — like wise via sympathetic fibers to the heart — does not accord with PERSS's obser vations in his investigation of 1958. He showed, however in a subsequent investigation (PERSS 1960) that it is possibl by pressor area stimulation to induce excitation of chronotropic fibers to the heart. The increased electrical discharge in the inferior cardia nerve observed by ALEXANDER (1946) on stimulation in the pressor area may — according to my findings — have been due to excitation of both cardio-accelerator and cardio-augmentor fibers.

The results of this investigation do not conflict w th the view that the pressor area is concerned in excitation of th entire sympathetic system (CHEN *et al.* 1936, 1937 a, b) Thus it was found that sympathetic vasoconstrictor nerves to muscle blood vessels, sympathetic accelerator and augmentor fibers to the heart, nd sympathetic secretion-activating nerves to the drenal medulla were

excited by stimuli in the pressor area. An increase of the adrenal medullary secretion on stimulation in that area of the medulla oblongata has been described previously (Chen *et al.* 1936, 1937b, Peiss 1958, among others). In this connection Chen *et al.* found it less likely that normally innervated and unpotentiated organs respond to the liberated catechols. Peiss, on the other hand, thought that with stimulation in the pressor area, catechol amines were released from the adrenals in quantities sufficient to produce distinct vasoconstriction and appreciably elevated cardiac activity. The present results corroborate Peiss's belief that the adrenal medullary secretion may be implicated in the augmentation of cardiac activity which accompanies stimulation in the pressor area. The extent, to which the liberated amounts of catechol amines comes into play in the physiological pattern of reaction, is however still unknown.

In an area of the ventrolateral portion of the medulla oblongata, coinciding with that through which the sympathetic vasodilator outflow passes, stimulation gave rise to excitation of inotropic and chronotropic fibers to the heart, apparently independently of each other, as was the case on stimulation of the vasodilator outflow in the hypothalamus (Rosén 1961a). All muscle vasodilator responses to stimulation in the ventrolateral portion were accompanied by an augmented cardiac activity. Since the vasodilator responses failed to occur on stimulation following atropinization, they had evidently stemmed from activation of the sympathetic vasodilator pathway. In some instances stimulation in that part of the medulla oblongata elicited — parallel with the elevated cardiac activity — no vasomotor change in the muscles. It does not necessarily follow however, that excitation of the vasodilator outflow had been absent in those experiments: the presence of a muscle vasodilator response could have been concealed by simultaneous vasoconstriction. Eliasson *et al.* (1951) thus showed that central stimulation in the vasodilator pathway might elicit — parallel with a vasodilator response — a vasoconstrictor effect in the muscles.

It was not previously known that stimulation in the "sympathetic vasodilator area" within the ventrolateral portion of the medulla oblongata causes augmentation of the cardiac contractile force. Peiss (1958, 1960) observed in his investigations the occurrence of tachycardia with a short latency on stimulation in the ventrolateral region of the medulla, and assumed that the heart rate was increased via activation of hypothalamo-spinal pathways. According to my observations it is probable that these stimulations affected the vasodilator outflow. It has previously been demonstrated that in the hypothalamic sector of the vasodilator system vasodilator fibers are accompanied by cardio-active fibers (Rosén 1961a). The present investigation shows that such fibers are also involved in the bulbar sector of the vasodilator outflow. According to my results the amount of adrenaline released on bulbar stimulation of the sympathetic vasodilator pathway was not sufficient to influence appreciably the vasomotor tonus in skeletal muscles. Together with the liberated noradrenaline it may however have sufficed to increase the cardiac activity. These two

findings confirm other observations (LINDGREN et al. 1959b, ROSÉN 1961b) The question, however still remains to which degree the catechol amines under physiological circumstances contribute to the increased cardiac activity

LINDGREN and UVNÄS (1955) showed that no anatomical or functional relationship exists between the vasodilator nerves and the pressor area. It has been suggested that the pattern of responses to stimulation of the vasodilator outflow might constitute a functional unit. The question then arises as to the physiologic significance of the vasodilator system.

The muscle vasodilatation and the cutaneous and intestinal vasoconstriction evoked by central stimulation of the vasodilator outflow (ELIASSON et al. 1951 LINDGREN and UVNÄS 1953 LINDGREN 1955) tend to produce a shift of blood volume to the skeletal muscles. Redistribution of blood to muscle tissue in order to establish optimal conditions for muscular effort takes place in states of emotional stress and, initially in muscular work. BLAIR et al. (1959) and ABRAHAMS et al. (1960) called further attention to the possibility already discussed by ELIASSON et al. (1951) and UVNÄS (1954) that the vasodilator system may be activated in emotional stress. Since the sympathetic vasodilator outflow has cortical origin (ELIASSON LINDGREN and UVNÄS 1952) it may be concerned with the initial adjustment of the muscle blood flow during exercise. That hypothesis, however is not in line with BLAIR et al. (1961) belief. The cardiac activity is increased both in emotional excitement and, initially in muscular exercise (for references see ROSÉN 1961a) My observations provide som evidence that the augmentation of cardiac contractile force and the increased heart rate together constitute a functional part of the response pattern of the vasodilator system. This lends further support to the hypothesis that the sympathetic vasodilator outflow is activated in situations such as fear anxiety unrest and other emotional states, as well as in the initial phase of muscular work, in order to establish suitable cardiovascular conditions.

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From the Department of Pharmacology Karolinska Institutet, Stockholm 60, Sweden

Cardiac Inotropic and Skeletal Muscle Vasomotor Responses to Adrenaline and Noradrenaline Threshold Doses on Intravenous Infusion

By

ANDERS ROSÉN

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Abstract

ROSÉN, A. *Cardiac inotropic and skeletal muscle vasomotor responses to adrenaline and noradrenaline. Threshold doses on intravenous infusion.* Acta physiol. scand. 1961. 53. 270—275. — In 1 cats under chloralose and urethane anesthesia, the mean threshold dose with standard deviation of intravenously infused adrenaline for cardiac inotropic response was found to be 0.26 ± 0.12 μg/kg/min and for muscle vasodilator response 0.44 ± 0.20 μg/kg/min. The mean threshold dose of noradrenaline for an inotropic response amounted to 0.37 ± 0.14 μg/kg/min and for a muscle vasoconstrictor response to 1.17 ± 0.45 μg/kg/min. The necessity of caution in estimating the differences in the threshold doses between inotropic and vasomotor responses is emphasized.

Activation of the sympathetic vasodilator outflow by intracerebral electric stimulation elicits — via the sympathetic innervation to the vessels of the skeletal muscles — vasodilatation in the muscles of the dog and cat. In the cat, there is a concurrent increase in the contractile force of the myocardium, produced via the sympathetic innervation to the heart (ROSÉN 1961 a, b). Furthermore central stimulation of the vasodilator pathway results in a raised output of adrenaline and noradrenaline from the adrenals (GRANT *et al.* 1958, LINDGREN, ROSÉN and UVNÄS 1959 a). On stimulation of moderate intensity the increase in either of these catechol amines is not, as a rule, sufficient to produce any appreciable vasomotor response in the muscles (LINDGREN, ROSÉN and UVNÄS

1959 b) Their raised output does, on the other hand, often suffice to elicit a marked increase in the contractile force of the heart (Rosén 1961 a, b) In the present investigation the threshold doses of intravenously infused adrenaline and noradrenalin required to elicit cardiac inotropic and muscular vasomotor responses were estimated. This was done to determine 1) if the excretion of catechol amines on central stimulation of the vasodilator outflow is — on comparison with the threshold doses for cardiac inotropic responses — sufficient to induce an increase in the contractile force of the heart 2) if there is a difference in the threshold dose of the catechol amines for a cardiac inotropic and a muscle vasomotor response.

Methods

The experiments were performed on 9 cats, weighing from 2.5 to 3.8 kg, anesthetized with chloralose (25—50 mg/kg) and urethan (200–500 mg/kg) Solutions of 1-adrenaline hydrochloride and 1-noradrenaline bitartrate in saline were prepared, and placed on ice. All doses are expressed as free base. A polyethylene catheter was inserted in branch of the left brachial vein. The catheter was connected to 20 ml syringe, mounted in constant infusion pump capable of delivering various calibrated infusion rates. In 3 animals, only one rate of infusion was used (1.8 ml/min) with variations in the concentration of catechols in the solution. I the remaining 6 animals, the concentration was constant (10 μg/ml) whereas the rate of infusion varied, but never exceeded 1 ml/min. The infusions were continued until the new levels in the recordings were stable for t least minute. After cessation of the infusion and the amplitudes in the recordings were back to the initial level another period of generally 2—4 min was allowed to pass until next infusion started. The increase of dose from one infusion to the next was in the range of 79—100 per cent. Due to spontaneous fluctuations 10 per cent increase in the contractile force was considered to be the threshold response. For the same reason 10 per cent decrease or increase in the peripheral resistance in the muscle vessels was considered to be the threshold vasodilator and vasoconstrictor response. The peripheral resistance (PR) was calculated according to the formula of Green *et al.* (1944) $\text{PR-unit} = \frac{1 \text{ mm Hg}}{1 \text{ ml/min}}$ The smallest infused amount producing the threshold response was considered to be the threshold dose.

For an account of determinations of the muscle blood flow arterial pressure, heart rate and contractile force of the heart, as well as of the relevant surgical techniques, reference is made to previous paper in this issue (p 255) In 7 experiments the contractile force of the heart was measured with the modified strain gauge arch (Rosén 1961) and in 2 experiments with the original type (Boniface *et al.* 1953)

Results

Infusion f adrenaline

Adrenaline augmented cardiac contractil force with a mean threshold dose of 0.26 μg/kg/min with standard deviation $\pm$ 0.12. The mean threshold dose for vasodilatation in skeletal muscle was 0 44 $\pm$ 0.20 μg/kg/min.

One of the experiments is illustrated in Fig 1 The series was started by infusion of 0 14 μg/kg/min of adrenaline (not included in the figure) This d

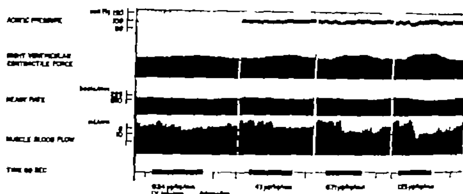


Fig. 1. Cat 3.5 kg. Aortic pressure, ventricular contractile force, heart rate and muscle blood flow responses to intravenous infusions of adrenaline.

produced no cardiovascular response. An increase in the contractile force of the right ventricle was, however elicited by the next infusion (0.24 μg/kg/min), which was considered to be the threshold dose for an inotropic response. No vasodilator response appeared on infusion of this dose, whereas this was the case with 0.43 μg/kg/min, which was considered to be the threshold dose for a vasodilator response. In another illustrated experiment, the threshold doses were the same for the two responses, 0.28 μg/kg/min (Fig. 2). In all experiments, higher doses — up to the largest quantity infused generally 1—2 μg/kg/min — still elicited vasodilatation in the muscles.

The heart rate was recorded in these two experiments, as in all the others. The response ranged from no increase in rate or a minimal one to a rise which, on infusion of quantities up to 1 μg/kg/min, seldom exceeded 10 per cent of the initial value.

Infusion of noradrenaline

In the 7 experiments with infusion of noradrenaline, no observations were made to indicate that any great quantitative difference is present between adrenaline and noradrenaline, as far as their effect on the contractile force of the heart is concerned. The mean threshold dose of noradrenaline for a cardiac inotropic response amounted to 0.37 μg/kg/min with standard deviation $\pm$ 0.14. Noradrenaline caused muscle vasoconstriction with a mean threshold dose of 1.17 $\pm$ 0.45 μg/kg/min.

The effects of a series of noradrenaline infusions are shown in Fig. 2. The increase in the contractile force of the heart was of the same extent as that produced by the corresponding quantity of adrenaline. In this series, the threshold dose for a vasoconstrictor response was 0.83 μg/kg/min. No increase in heart rate was recorded and in the remaining experiments that was in general also the case.

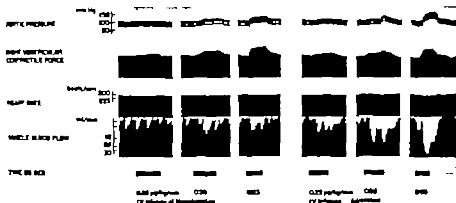


Fig. 2. Cat 3.0 kg. Aortic pressure, ventricular contractile force, heart rate and muscle blood flow responses to intravenous infusions of noradrenaline and adrenaline.

Discussion

The present investigation showed that intravenous infusion of adrenaline as well as of noradrenaline in a threshold quantity of 0.1—0.5 μg/kg/min produced an increase in the contractile force of the heart in the cat. It thus confirmed the observations of COTTEN and PINCUS (1955) and WEST and RUSHMER (1957) among others, that no great quantitative difference is present between the cardiac inotropic effect of adrenaline and noradrenaline. The literature contains very few quantitative data on the effect of intravenously infused catechol amines on the contractile force of the heart. WEST and RUSHMER found that intravenous infusion of 0.001 μM/kg/min of both adrenaline and noradrenaline produced an increase in the stroke work of the ventricle in the dog. That value, however can not be compared with those, obtained in the present investigation. I found that infusions of adrenaline elicited slight to moderate tachycardia. Infusions of noradrenaline, on the contrary generally resulted in no change in heart rate. These findings are largely in agreement with those of WEST and RUSHMER in the dog.

On hypothalamic excitation of the vasodilator pathway by stimuli of moderate intensity (0.5—2.5 V) with frequency 70/sec, GRANT *et al.* (1958) found the mean value for the output of adrenaline from one adrenal to be 0.23 μg/kg/min. The corresponding figure for noradrenalin was 0.50 μg/kg/min. On stimulation of the vasodilator pathway in the medulla oblongata with the same parameters, the discharge of adrenaline from one adrenal amounted to 0.20 μg/kg/min and that of noradrenaline to 0.47 μg/kg/min (LINDGREN *et al.* 1959 a). If these figures are doubled to obtain the value for both adrenals, the excretion of catechol amines on the performed stimulations of the vasodilator outflow seems — on comparison with the threshold doses for cardiac inotropic responses re

corded in the present study — to be amply sufficient to induce an increase in the contractile force of the heart. This observation may explain why adrenalectomy often markedly reduces the increased cardiac responses to central activation of the vasodilator outflow (Rosén 1961 a, b).

In addition vasodilatation in the muscles was found to be produced by adrenaline in the mean threshold dose of 0.44 µg/kg/min with standard deviation ± 0.20. Noradrenaline, on the other hand, was unable to elicit vasoconstriction in the muscles until the dose reached 1.17 ± 0.45 µg/kg/min. These threshold doses are essentially in agreement with earlier observations (Celander 1954, Lindgren *et al.* 1959 b, and others). Lindgren *et al.* found that the quantity of adrenaline and noradrenaline released by hypothalamic and medullary stimuli of moderate intensity applied to the sympathetic vasodilator pathway was, in fact, too low to exert any appreciable vasomotor action. My observations have also led to this conclusion.

Using a paired t test the p-value for the difference between the threshold dose of adrenaline, as well as of noradrenaline, for a cardiac inotropic and a muscle vasomotor response was < 0.01 but > 0.001. The question of whether this small difference is physiologically significant cannot be definitely answered. The two responses are indeed dissimilar, that is, the contractile force measurement represents a more direct parameter than does peripheral resistance, and furthermore, one cannot have confidence in quite comparable local tissue concentrations.

On electrical stimulation of the sympathetic vasodilator system, the quantity of catechol amines liberated from the adrenals has a greater effect on the contractile force of the heart than on the vasomotor tonus of the muscles. This is apparently due to the low threshold dose of adrenaline and noradrenaline required for a cardiac inotropic response.

The conceivable physiologic significance of the vasodilator system has been discussed in earlier publications (Eliasson *et al.* 1951, Uvnäs 1954, Abrahams *et al.* 1960, Uvnäs 1960, Rosén 1961 a, b). Here, it suffices to recall the possible implication in situations of emotional stress (Abrahams *et al.* 1960) and in the initial stage of muscular work.

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Acta physiol. scand. 53. 1961 276—287

From the Department of Physiology, Karolinska Institutet, Stockholm, Sweden

The Effect of Acetylcholine and Related Substances on the Isolated Muscle Spindle

By

D. Ottoson

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Abstract

Ottoson, D. *The effect of acetylcholine and related substances on the isolated muscle spindle.* Acta physiol. scand. 1961. 53. 276—287. — In studies on the action of various drugs on the isolated frog's muscle spindle it was found that ACh in concentrations below 1×10^{-4} had no significant effect on the sensory endings of the spindle, while higher concentrations (5×10^{-4}—1×10^{-3}) caused a slight reduction of their activity. Other cholinesters like butyrylcholine, propionylcholine and succinylcholine had a similar action as ACh. Nor-acetylcholine in concentrations of 5×10^{-4}—1×10^{-3} caused a suppression of the spontaneous activity and a reduction of the discharge during stretch. Of the anticholinesterases that were studied eserine, DFP and mintacol had an excitatory action that was followed by a cessation of the spontaneous discharge and a block of the response to stretch. Eserine was active in concentrations of 2×10^{-4}—1×10^{-3}, DFP in concentrations of 3×10^{-4}—1×10^{-3} and mintacol in concentrations of 5×10^{-4}—1×10^{-3}. Prostigmine had no significant action on the spindle. Histochemical determination of the cholinesterase activity in the spindle showed the presence of slight concentrations of cholinesterases in the polar regions of the spindle.

As shown by Katz (1950) stretch sets up in the muscle spindle a local potential which secondarily gives rise to a discharge in the sensory axon. The development of the receptor potential can not be explained as due to an increase in the capacity of the membrane although such a mechanism may be involved in the production of the dynamic component of the response (Katz 1950). The suggestion has therefore been made that the deformation of the sensory membrane causes permeability changes with a subsequent ionic flow. These

changes can be assumed to arise either as a direct result of the mechanical distortion of the membrane or they may be mediated through the release of some chemical transmittor (KATZ 1961) This last alternative raises the question of whether or not ACh is involved in the initiation of the receptor response.

The present paper describes some of the results of a study on the action of ACh and related substances on the activity of the isolated muscle spindle of the frog. One reason for using the isolated spindle was that in this preparation the test substances can be applied directly to the sensory endings. Another advantage lies in the fact that indirect effects which may confuse the interpretation of the results obtained in non-isolated preparations are absent or can easily be eliminated in the isolated spindle. Furthermore, since the receptor potential can be recorded together with the impulse discharge, it is possible to get clues as to the site of action of an applied substance.

The results obtained show that none of the quaternary ammonium compounds that were tested produced excitation whereas some anticholinesterases gave rise to an intense discharge from the spindle.

Methods

Preparation. The experiments were made on isolated spindles of *m. ext. dig. long. IV* of the frog This muscle contains —3 spindle systems each of which consists of a bundle of intrafusal muscle fibres with the spindles arranged in series (GRAY 1957) The anatomical arrangement of these systems is usually very constant and the spindles are therefore easy to find. The dissection was carried out under dark field illumination with the muscle mounted in Ringer bath The first step was to locate spindle and then to isolate the nerve bundle running to it. This bundle contains besides the sensory axon a number of motor fibres to the intrafusal muscles. The motor fibres sometimes follow the sensory axon to its entrance through the spindle capsule and from here they run along the spindle to the inter-sensory portions of the intrafusal muscles. In other cases the motor fibres leave the sensory axon t some distance from the spindle. All the fibres except the sensory axon were severed and the spindle cleared from adjacent tissue. The intrafusal bundle was then cut close to the ends of the spindle and the preparation lifted over into smaller chamber (0.5 × 2 mm) where it was clamped at its ends to thin nylon rods (0.15 mm diam.) One of these rods was fixed to the wall of the chamber while the other was connected to an electromagnet by which stretches could be applied. The electromagnet was mounted on to micromanipulator so that the resting length of the spindle could easily be changed.

It was often observed that the intrafusal fibres in the mounted preparation showed slow tonic contractions or fast fibrillating twitches which produced a discharge from the spindle. This occurred most frequently when th intrafusal bundle had been cut at some distance from the spindle When testing the effect of various compounds on the sensory endings it was essential t eliminate secondary effects caused by the action of the drugs on the intrafusal muscles. These were therefore alway killed by pinching them as close as possible to the ends of the spindle I addition it was sometimes found necessary to puncture the intracapsular portions of the intrafusal fibres with a coarse (2—5 μ) glass-capillary

Recording Recordings were made with Ag—AgCl electrodes connected to the preparation with glass tubes filled with Ringer Agar One electrode was placed in the Ringer

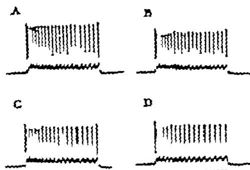


Fig. 1 The effect of acetylcholine on the stretch response. A, in Ringer B—D 5, 10 30 min after application of ACh (1 × 10⁻⁴) Time 100 msec.

solution in the chamber while the other was applied to the sensory axon, which as lifted up in oil. The Ringer-oil interface was at the portion of the sensory axon where it enters the capsule. The electrodes were connected to a direct coupled amplifier

Electrical artifacts which were difficult to distinguish from the receptor potential were sometimes recorded when the spindle was stretched. Tests showed that these artifacts could not be explained as caused by the grid current (1 × 10⁻ A) solely I many cases they could be traced back to the presence of a polarization oltage t the recording electrodes. Therefore electrodes to be used were always first tested on killed preparation to ensure that they gave no such artifacts.

Solutions The following drugs were tested acetylcholine chloride butyrylcholine iodide, propionylcholine chloride, nor-acetylcholine (β-acetoxy-ethyl-dimethyl-dodecyl ammonium chloride prostigmine, eserine salicylate, di-iso-propylfluro phosphonate (DFP) p-nitrophenyl-diethylphosphate (mintacol) nonamethonium iodide tubocurarine chloride and hexamethonium bromide. The solutions were always freshly prepared for each experiment. The concentrations are expressed in g/ml. The Ringer solution containing the drug to be tested was applied to the spindle after the fluid in the chamber had been partly removed with a pipette. Removal of all the fluid in the bath was not practical since this resulted in a positional change of the spindle and discharge. Great care had therefore always to be taken so that the change of solution did not stretch the spindle. In order to replace all of the Ringer solution in the chamber with the test solution several changes of fluid had to be performed. This could usually be done in less than one min. To ensure that during this procedur no unusual effects were overlooked all drugs were also tested by adding a small drop of solution of high concentration to the bath so that the drug may reach the spindle by diffusion

Results

When the spindle is kept at resting length t usually fires at a low rate. This discharge goes on for hours with no appreciable change in frequency and seems to be a sensitive index of the functional state of the spindle It was often observed that drugs in concentrations which had no significant effect on the response to stretch produced a change in the spontaneous discharge rate, thus revealing that they ere not entirely without effect

The test stretches used in the present study were usually adjusted so as to give a moderate response. As a rule this response remained constant for periods

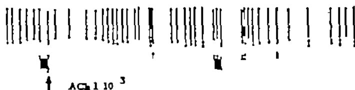


Fig. 2. The absence of effect of acetylcholine on the spontaneous activity of the muscle spindle.

of several hours provided the spindle was not stimulated too frequently. However within the first 10—15 min after the spindle was mounted the response to a given stretch often varied a great deal. The spindle was therefore always left to rest for about 15 min after being mounted, and no tests of the effects of drugs were done before the response had been constant for at least 15 min.

The effect of cholinesters

ACh in concentrations below 1×10^{-4} generally had no conspicuous action on the spindle. In concentrations of 5×10 —1×10^{-4} the drug caused a decrease in frequency of the spontaneous discharge. This change was usually very slight, but in some preparations th spindle became silent after having been soaked in the test solution for a few minutes. In concentration of 1×10^{-3} ACh usually also caused a reduction of the stretch response. This change took place slowly and did n t become marked until the spindle had been exposed to the drug for 20—30 min. In many preparations which appeared to be in perfect condition ACh had very little effect, as is illustrated in Fig 1. It must be emphasized that ACh in concentrations of 1×10^{-7}—1×10^{-3} was never observed to produce any initial excitatory action (Fig 2). This lack of effect could not be due to an inability of ACh to pass through the spindle capsule, since identical results were obtained after the spindle capsule was split.

It is well known that ACh even in high concentrations is inactive on myelinated nerve fibres. The absence of effect has been explained as being due to the failure of ACh to reach the inside of the membrane (NACHMANSOHN 1959). Recently a number of lipid soluble quaternary ammonium compounds have been developed, which are reported to depolarize myelinated nerve fibre (STÄMPFLI 1958, DETTBARN 1959). One of these, referred to as nor ACh, was tested on the spindle during the present study. It was found that this compound in concentrations of 5 10^{-3}—1 10^{-4} caused a pronounced decrease in the spontaneous activity of the spindle. This effect took place within a few seconds after application of the drug and often led to a cessation of the spontaneous discharge within 1—5 min. If the solution in the bath was not stirred or renewed, it sometimes occurred that the spindle after having been silent for som minutes started to fire again at a slow rate. Renewal or stirring of the solution immediately stopped this discharge. The response to

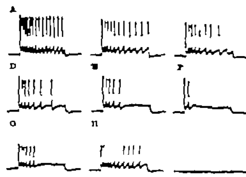


Fig. 3 The effect of nor-acetylcholine A, in Ringer B—F after 1, 3, 6, 10 and 12 min in nor-ACh (3×10^{-4} w/v). G—H, recovery after 10 and 30 min in Ringer I, after the spindle had been punched. Time 100 msec.

stretch also became reduced although this effect developed less rapidly than the decrease of the spontaneous discharge. Thus, stretches produced responses long after the spindle had ceased to fire spontaneously As illustrated in Fig 3 the effect of nor-ACh on the stretch response appears as a progressive decrease in the discharge until only a few impulses are left. The remaining impulses are relatively resistant to the action of the drug but finally drop out after prolonged exposure. It may be noted that the action of the drug on the sensory endings takes place more slowly than that on the axon as is revealed by the fact that the receptor potential remains almost unchanged at a stage when most of the nerve impulses are extinguished (record F) If the preparation was rinsed in Ringer before the response had disappeared entirely the activity of the spindle was partly restored (records G and H) Nor acetylcholine was also tested together with subthreshold concentrations of eserine ($1—2 \times 10^{-6}$) The effect obtained was essentially the same as with nor-acetylcholine alone.

Of the other cholinesters that were tested butyrylcholine and propionylcholine had a similar action as ACh but were slightly less potent. Neither of them produced any excitatory effect It has been demonstrated that succinylcholine has a stimulating action on non-isolated spindles (GRANIT *et al.* 1953, HENATSCH and SCHULTE 1958) In the isolated spindle of the frog a similar effect could not be obtained In concentrations below 1×10^{-4} succinylcholine did not affect the spindle whereas higher concentrations ($2—5 \times 10^{-4}$) produced a slight reduction of the spontaneous discharge as well as of the response to stretch.

Anticholinesterases

The anticholinesterases studied in the present investigation were prostigmine eserine, DFP and mintacol

No readily detectable change in the activity of the spindle could be obtained with prostigmine in concentrations as high as $5 \times 10^{-4}—5 \times 10^{-4}$ (Fig. 4) In some preparations a decrease in the spontaneous activity occurred and the response to stretch became slightly reduced These effects appeared slowly

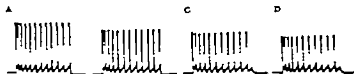


Fig. 4. The effect of prostigmine. A, in Ringer B—D after 1 5 and 15 min in prostigmine (1 × × 10^{-4} w/v). Time 100 msec.

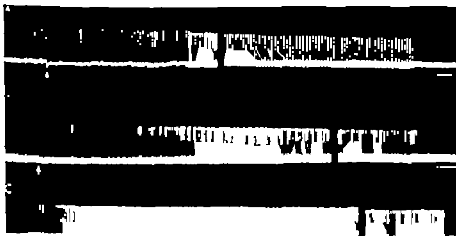


Fig. 5 A. The effect of eserine (5 10^{-4} w/v) Application of one drop of the solution (arrow) causes discharge lasting for about 30 sec and ending abruptly Time 1 sec.

B. The effect of DFP One small drop of DFP (5 × 10^{-4}) pplied in the bath (arrow) Time 1 sec.

C. The effect of mintacol. One small drop of mintacol (1 × 10^{-4} w/v) applied in the bath about 5 sec before the recording was started. Note negative d. c. shift with increasing firing frequency Time 1 sec.

and did often not become marked until after long (30—45 min) exposures. It could therefore not be ascertained whether or not these changes could be ascribed to the action of the drug

Eserine in concentrations of 2 × 10^{-4}—5 × 10^{-4} usually produced an initial excitatory effect (Fig 5 A) followed by a subsequent reduction of the sensitivity of the spindle to stretch and finally a complete block. The excitatory effect varied a great deal from on spindle to another In some preparations there occurred a vigorous discharge that started 1—3 sec after application of the drug and usually lasted for 20—40 sec. Most often the firing ceased suddenly and the spindle thereafter remained silent. As with nor ACh a certain recovery sometimes occurred if the solution was not stirred or renewed (*cf* EDWARDS and KUFFLER 1959) In other preparations eserine only caused an inconspicuous

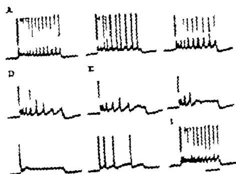


Fig. 6. The effect of eserine. A, in Ringer. B—G after 1, 3, 4, 5, 6 and 10 min in eserine (5×10^{-4} w/v). H—I, 1 and 3 min after washing with Ringer.

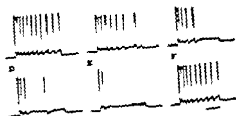


Fig. 7. The effect of DFP on the stretch response. A, in Ringer. B—E after 1, 2, 3 and 4 min in DFP (2×10^{-4} w/v). Record F 2 min after washing with Ringer.

increase of the activity. It was also observed that application of the compound in high concentrations (1×10^{-3}) sometimes caused a sudden cessation of the spontaneous activity without any preceding period of increased firing.

The response to stretch was increased during the period of intense firing whereas after the spindle had become silent a rapid decrease occurred. As seen in record B—G in Fig. 6 the discharge during stretch was diminished in a way reminiscent of the effect produced by nor-ACh. It may be noted that in the early stage of the block there appear abortive spikes (B) and non-propagated prepotentials (E and F). As the impulses gradually drop out, the receptor potential becomes uncovered and is finally obtained almost isolated (G). The blocking effect on the discharge is reversible, and the original activity is restored within a few minutes after the preparation is rinsed in Ringer (I).

In concentrations of 3×10^{-4}—1×10^{-3} DFP produced an intense discharge that began a few sec after the test solution was applied. As seen in Fig. 5 B there occurs at the same time a negative d. c. shift, indicating a depolarization of the spindle. The excitatory effect usually lasted not more than 10—20 sec and most often the discharge stopped abruptly. The ensuing reduction of the excitability took place considerably faster than with eserine and sometimes led to a complete abolition of the impulse discharge within a few sec. Stretches applied immediately after the discharge had stopped often gave only one or a few impulses superimposed on a barely detectable local potential. With threshold concentrations the blocking effect took place less rapidly and the de-

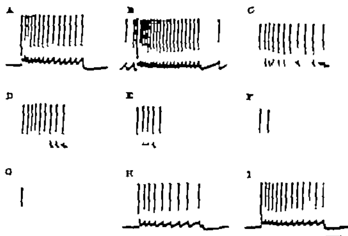


Fig. 8. The effect of mintacol on the stretch response. A, in Ringer B—G, after 1/2, 1 1 1/2, 2 3 and 5 min in mintacol (5×10^{-4} w/v) Record B was obtained during the period of intense firing. C—G after the spindle had become silent. Records H—I, 2 and 3 min after washing with Ringer Time 100 msec.

velopment of the block could be studied. As seen in Fig 7 the effect is similar to that caused by eserine. The impulse discharge is again more sensitive to the action of the drug with the result that the receptor potential is left partly uncovered. However this potential also undergoes a rapid reduction and is abolished if the preparation is exposed to the drug for some minutes. In the experiment illustrated in Fig 7 the preparation was washed in Ringer after record E was obtained. As seen in record F obtained 2 min later the spindle regains its excitability rapidly

Mintacol had an effect much like that of eserine and DFP In threshold concentrations of 3×10^{-4}—5×10^{-4} the compound caused an increase in the spontaneous activity and a sensitization of the spindle to stretch. This effect usually vanished within a few minutes but could sometimes be maintained for 10—15 min by repeated changes of the solution in the bath. In higher concentration (1×10^{-3}) mintacol produced a vigorous discharge lasting for 30—40 sec (Fig 5 C) The sensitivity to stretch was increased during this period as is illustrated by record B (Fig 8) After the spindle had ceased to fire there followed a fast reduction of the response (C—G) It may be noted that this change also involved a rapid diminution of the receptor potential. The effect caused by mintacol was usually reversible, and the activity was rapidly restored if the spindle was rinsed in Ringer (record H—I Fig 8) It was a constant observation that a second application of the drug shortly after the activity had been restored with a rinse of Ringer failed to produce any effect or only gave rise to a short burst of impulses Only if the concentration was raised could the earlier



Fig. 9 The effect of tubocurarine, A, in Ringer B—D after 10, 20 and 30 min in tubocurarine (3×10^{-4} w/v) Time 100 msec.

excitatory effect be obtained again. The same finding was made with DFP The excitatory effect of eserine, DFP and mintacol was not obtained after the spindle had been pinched.

Blocking agents

Tubocurarine in concentrations of 1×10^{-6}—1×10^{-4} as a rule failed to produce any significant effect. When the concentration was raised (3×10^{-4}) a slight reduction of the response and of the spike height was sometimes obtained (Fig 9) However this effect often did not become detectable until after comparatively long exposures. Hexamethonium in concentration of 1×10^{-4} had an action similar to that of curare.

Cholinesterase-staining of the spindle

In connection with the study of the action of the different compounds described above a number of spindles were also stained according to the method developed by HOLMSTEDT (1957) It was found that no staining occurred in many of the preparations while others showed a slight positive AChE reaction. When staining occurred it was always confined to the polar regions of the spindle (Fig 10) This finding is consistent with earlier observations (COERS and DURAND 1956, GIACOBINI and HOLMSTEDT 1960)

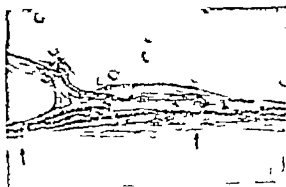


Fig. 10. Cholinesterase staining of isolated spindle Note slight positive reaction at polar ends (arrows)

Discussion

As was first shown by CAJAL in 1888 the sensory fibre of the muscle spindle breaks up into fine unmyelinated strands with numerous varicosities distributed along the intra-capsular portion of the intrafusal fibres. According to KATZ (1961) who quite recently has published a report on the electron-microscopic structure of the frog's spindle, these varicosities consist of bulbous expansions which are 2—3 μ thick and connected through thin (0.15 μ in diam.) tubes. There are reasons to believe (KATZ 1961) that these bulbs form the mechano-electrical transducers in the spindle system. The first step in the electrical event leading to the sensory discharge must accordingly occur in the membrane of these structures. It is not possible to decide at present whether or not this first step involves the release of some active substance. It has been found in studies on curarized frog's whole muscles that ACh excites the spindle (HENATSCH and SCHULTE, 1958). In the cat the spindle response to intravenous injection of ACh is blocked by prior administration of tubocurarine (HUNT 1952). No excitatory action was obtained in the present study when ACh was applied directly on the isolated spindle. The lack of effect can not be explained by the assumption that the spindle capsule offers a diffusional barrier since splitting of the capsule did not alter the experimental result. However the failure of ACh to excite the isolated spindle can not be considered as a conclusive evidence against the ACh-theory since the receptor membrane may be impermeable to the compound. On the other hand the fact that nor ACh, which is supposed to penetrate the membrane, also failed to excite the spindle seems to make it unlikely that ACh or any related substance is involved in the excitatory process.

In striking contrast to the relative ineffectiveness of the cholinesters stands the powerful action of the anticholinesterases. With the exception of prostigmine they all had essentially the same action an initial excitatory effect and a subsequent reduction of the activity of the spindle. The fact that prostigmine did not cause these effects is most likely to be ascribed to the failure of the compound to penetrate lipid membranes. It has been demonstrated that prostigmine in concentrations as high as 0.01 M leaves conduction in nerves unaffected (BULLOCK *et al* 1946). Eserine on the other hand is known to pass readily through the nerve membrane. It blocks conduction when applied externally to nerves (BULLOCK *et al* 1946, LORENTE DE NÓ 1947, DETTBARN 1959, WRIGHT 1956). This effect is not due to a depolarization, since eserine does not reduce the resting potential of the nerve (LORENTE DE NÓ 1944, STRAUB 1953). DFP gave an effect very much like that of eserine but was more potent as judged from the finding that it caused a more rapid and intense effect. This can most likely be ascribed to the fact that DFP is more lipid than water soluble.

The excitatory effect of eserine, DFP and mintacol raises the question as to the mode of action of these substances. Th main argument against the idea that they excite the spindle by blocking the cholinesterase activity is the finding that

neither ACh nor any other of the quaternary ammonium compounds that were tested produced excitation. Furthermore the finding that the spindle contains no or only very small amounts of cholinesterase suggests that the excitatory effect of the anticholinesterases may not be mediated by the ACh system. This view is also supported by the observation that eserine and DFP had to be used in relatively high concentrations. It has been demonstrated that anticholinesterases also may affect other enzyme systems (MICHAELIS *et al.* 1949). The possibility therefore exists that the effect of eserine and DFP on the spindle is unspecific. The fact that mintacol is active in concentrations as low as 3×10^{-6} suggests, however, that this compound may have a specific action. Whatever the explanation may be as to the precise mode of action of the inhibitors, the findings suggest the existence of an enzymatic system involved in the processes maintaining the membrane potential of the sensory endings.

The effect of anticholinesterases has been studied in many different types of sensory end organs. In most of them no effect has been found while in a few types an increased activity has been demonstrated. Thus, it has been shown that eserine augments the activity of the chemoreceptors of the cat's carotid sinus (LANDGREN *et al.* 1952). It has also been demonstrated that prostigmine increases the activity of the taste fibres in the cat (LANDGREN *et al.* 1954). One may ask why these compounds do not have similar effects on other types of sensory end organs. The explanation to this seems to be that the inhibitors have to reach the active sites in sufficiently high concentrations within a limited duration of time. Sensory endings which are surrounded by tissues acting as diffusional barriers will only be gradually reached by an externally applied drug. The probability that the excitatory effect will be obtained is therefore greatly increased in isolated preparations or in preparations where the receptors are easily reached by the drug.

It has been pointed out that there is very little likelyhood that ACh participates in the generation of the response of sense organs to their physiological stimuli (GRAY and DIAMOND 1957). The results obtained in the present study indicate that this may also be true for the spindle although the effect of the anticholinesterases suggests the possibility that chemical processes may be involved in the initiation of the discharge.

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From The Institute for Medical Biochemistry and Physiology Department of Physiology
University of Oslo, Norway

So-called Free Erythrocyte Protoporphyrin and its Possible Role in Hemoglobin Formation

By

LEIF ERIKSEN

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Abstract

ERIKSEN, L. *So-called free erythrocyte protoporphyrin and its possible role in hemoglobin formation.* Acta physiol. scand. 1961. 53. 288—299. — The relations of so-called free erythrocyte protoporphyrin to the number of reticulocytes in the blood of rabbits made anemic by daily injections of 25 mg phenylhydrazine for 5 days, has been studied. The heme forming capacity of the ed cells as related to the number of reticulocytes has also been studied. All the data obtained are compatible with the assumption that the amount of free protoporphyrin is not strictly related to the number of the reticulocytes but to the stage of maturation of these cells. The possibl role of free erythrocyte protoporphyrin in hemoglobin formation has also been studied, and it has been shown that the main pool of protoporphyrin is not normally utilized for heme formation. It is suggested that free protoporphyrin, although probably a normal intermediate in heme formation, when formed in excess diffuses out into the soluble part of cytoplasm and becomes unavailable for heme formation. The possibility that the true proto-type intermediate in heme formation differs chemically from protoporphyrin IX is, however not excluded. It is suggested that excess formed protoporphyrin may in part be the origin of the so-called early bile pigment fraction found in mammals.

It has long been known that the erythrocytes in the peripheral blood of mammals do contain small amounts of free protoporphyrin (VAN DEN BERG, GROTEPASS and REVERS 1932). SEGGEL (1940) was able to show that the porphyrin was present in the cells as a red fluorescing compound with a fluorescence spectrum identical with that of protoporphyrin. SEGGEL also presented evidence that the number of protoporphyrin containing cells, the so-called fluorocytes, increased in anemia and conditions characterized by an increased

inflow of young red cells into the peripheral blood. WATSON and CLARK (1937) first assumed that the protoporphyrin containing cells were the reticulocytes. However WATSON later partly withdrew this assumption suggesting that the fluorocytes although young cells, were not identical with reticulocytes (WATSON, GRINSTEIN and HAWKINSON 1944) SCHWARTZ and WIKOFF (1952) in keeping with the later results of Watson and co-workers found no correlation between the amount of free protoporphyrin and the number of reticulocytes in the peripheral blood. However neither WATSON et al. nor SCHWARTZ and WIKOFF did control that the population of reticulocytes studied actually could be compared, and since it has been shown by a series of authors that the reticulocytes may exist in the blood up to 4 days (for reference see SEIP 1953) it might very well be that the age of the reticulocyte may be the factor of importance in these studies and not the actual number of reticulocytes.

The possible role of the free erythrocyte protoporphyrin has long been debated, and it has become more and more probable that it is in some way directly related to hemoglobin formation. GRANICK (1954) presented indirect evidence that protoporphyrin added to or accumulated in young erythrocytes can be utilized for heme formation. Since then a series of authors (ERIKSEN 1956, KREUGER, MELNICK and KLEIN 1956, MINAKAMI, YONEYAMA and YOSHIKAWA 1958, NISHIDA and LABBE 1959) have shown that added protoporphyrin is readily utilized for heme formation in a series of different systems. However DRESEL and FALK (1956) found that unlabelled protoporphyrin added to chicken hemolysates was unable to dilute the incorporation of C into heme from different protoporphyrin precursors. They concluded that protoporphyrin similar to uroporphyrin and coproporphyrin does not lie on the pathway to heme but is a side product.

Since protoporphyrin is relatively insoluble at the pH used by DRESEL and FALK, their finding might be due to incomplete mixing of the protoporphyrin added and that formed by the hemolysate. However in view of our own finding that the non-heme protoporphyrin of rabbit reticulocytes was present in the cells in at least two metabolically different fractions (ERIKSEN 1955a, 1956) the possibility arose that the two protoporphyrin fractions found were not, as interpreted at the time, two steps in the normal formation of heme, but that only one was an intermediate and that the other was a side product differing from the true intermediate either in its chemical properties or in its localization within the cell or both.

The present investigation was undertaken to get more information on the so-called free erythrocyte protoporphyrin and its role in hemoglobin formation.

Material and methods

In the in vivo experiments 1-year-old rabbits of the laboratory stock were used.

In all in vitro experiments blood with reticulocyt numbers of 60—70 per cent were obtained from rabbits made anemic by daily subcutaneous injections of 25 mg phenylhydrazine for 5 days and bled to death on the 7th day. Cells and plasma were separated

by centrifugation for 15 min at a speed of 2,000 r. p. m. The cells were washed once with 0.9 % NaCl and finally suspended in two volumes of 0.9 % NaCl. This suspension was used as a stock suspension in all the incubation experiments described below except where otherwise has been stated.

As tracer substances were used acetate labelled in the carboxyl group (1 mc/mole) or the methyl group (10 mc/mmole) with ^{14}C, and ferric chloride labelled with ^{59}Fe (50 mc/g Fe). The radioactive substances were obtained from Atomic Centre, Amersham, England.

Protoporphyrin and hemin were extracted from the reticulocytes with ethyl acetate and glacial acetic acid 4 : 1. The free porphyrins were brought out with 3 N HCl, fractionated and purified in the usual way (Eriksen 1956). The amount of free protoporphyrin was estimated according to Grinstein and Wintrobe (1948). The identity of protoporphyrin was established by means of spectrophotometry and paper chromatography (Eriksen 1953). The specific activity of free protoporphyrin was measured as described elsewhere (Eriksen 1956).

Hemin was brought out of the ethyl acetate extract with 10 % ammonia after complete removal of all free porphyrins with 3 N HCl. The hemin was precipitated by acidification with small amounts of concentrated HCl, washed with 2 N HCl and distilled water, purified, plated and counted as described in detail elsewhere (Eriksen 1955 b, 1956).

In some experiments in which ^{59}Fe was used as tracer for the labelling of heme, the hemin prepared as described above was digested and the iron plated electrolytically and counted as described elsewhere (Eriksen 1955 b, 1956).

The reticulocyte counts were not performed directly on smears stained with brilliant cresyl blue, but after washing with methanol and restaining with diluted Giemsa solution. This was done since it was found that the direct technique gave too high values in phenylhydrazine poisoning (Eriksen and Jacobsen 1960).

Experimental and results

The relation of free protoporphyrin to the number of reticulocytes. 8 rabbits were injected subcutaneously with 5 mg phenylhydrazine for 5 days. The number of reticulocytes and the amount of free protoporphyrin were measured each day at 10 o'clock in the morning prior to a new injection. Since Schwartz and Wikoff (1952) found no correlation between the number of reticulocytes and the amount of free protoporphyrin, but a correlation factor of approximately 1 for coproporphyrin and the number of reticulocytes, we also measured the amount of the 0.1 N HCl extractable porphyrin which was present in the cells in small amounts. This porphyrin was not tested for purity by paper chromatography, however, the porphyrin was insoluble in chloroform, was readily brought out of ether with 0.1 N HCl, and the spectral absorption in the Soret band was identical with coproporphyrin, so there is good reason to believe that we actually are dealing with coproporphyrin.

As seen from Fig. 1, there is a good correlation between the number of reticulocytes and both protoporphyrin and coproporphyrin, with a maximum for all of them on the 7th day. However the increase in the number of reticulocytes from the 6th to the 7th day is far less than the increase in free protoporphyrin and coproporphyrin.

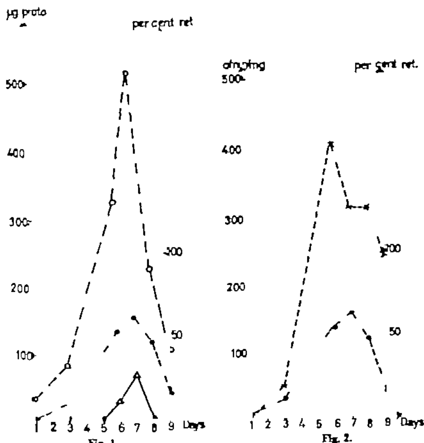


Fig. 1 The relation of free erythrocyt protoporphyrin and coproporphyrin to the number of reticulocytes in the blood of rabbits made anemic by daily injections of 25 mg phenylhydrazine for 5 days.
•— —• per cent reticulocytes, ———• µg protoporphyrin/100 ml cells,
△———△ µg coproporphyrin/100 ml cells.

Fig. 2. Heme forming capacity of red cells from anemic rabbits as related to the number of reticulocytes. The red cells were suspended in 2 vols 0.9 NaCl/glycine (0 03 M) and incubated aerobically with ^{14}C-acetate as tracer
•— —• c/min/mg hemin, •— —• per cent reticulocytes

Heme-forming capacity of ed cells from anemic rabbits as related to the number of reticulocytes Rabbits no. 1 to 6 were killed on the 1st, 3rd, 6th, 7th, 8th and 9th day The washed red cells vere suspended in 0 9 % NaCl/glycine (0.03 M) and incubated in air for 1 hour w th labelled acetat as tracer and the specific activity of hemin was measured As seen from Fig 2, th maximum specific activity is reached on the 6th day while the maximum of reticulocytes is reached on the 7th d y The relatively slow drop in the specific activity of hemin shows that the ability to synthesize hem from simple building stones

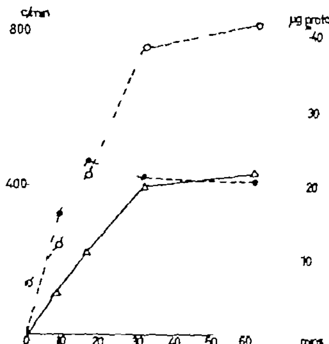


Fig. 3. The formation of free protoporphyrin and the incorporation of ^{14}C-acetate into protoporphyrin and heme by rabbit reticulocytes suspended in 2 vols. 0,9 % NaCl glycine (0,03 M) and incubated in air
•— —• μg protoporphyrin,
•– – –• /min/μg protoporphyrin,
△———△ c/min mg hemin.

such as acetic acid is not specifically linked to cells having a substancia granulofilamentosa" but is a property of the red cell up to a certain stage of maturation. So far we have no morphological means to identify these cells, however it might be that if the reticulocytes were classified according to HEILMEYER (1931) and correlated to the above parameters, one would be able to classify them according to their age and biochemical properties, and thus get some morphological reference for the biochemical and physiological properties of the red cells in their early phase of maturation It seems quite clear from the above results that the number of reticulocytes could not be used as a reference neither for the amount of free protoporphyrin nor for the ability to synthesize heme.

The utilization of preformed protoporphyrin for heme formation in whole cell systems in vitro The possible fate of the protoporphyrin accumulated in the young cells is so far unknown. It might be that it is a normal precursor to heme and is utilized for hemoglobin formation in the organism. Such an assumption is supported by the finding of LONDON, SHEMIN and RITTENBERG (1950) indicating that hemoglobin formation continues in the reticulocytes in the peripheral blood of the rabbit. Similar findings have been reported by REIZENSTEIN and OGATA (1957) in the rat.

However since the reticulocytes are fully capable to synthesize both the porphyrin and the globin part of hemoglobin from such simple building stones

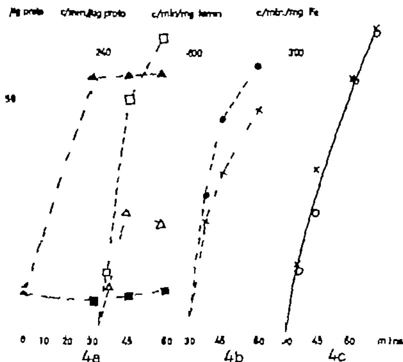


Fig 4 a, b, c. The incorporation of ^{14}C-acetate into protoporphyrin and heme and ^{59}F into heme by rabbit reticulocytes suspended in 2 vols. 0.9 % NaCl/glycine (0.03 M) and incubated in air. The cells had been preincubated aerobically in 0.9 % NaCl with and without addition of glycine for 30 min prior to the addition of the tracer substances.
Glycine series ▲— —▲ μg proto, △— —△ c/min/μg proto, •— —• c/min/mg ^{14}C-hemin, •——• c/min/mg Fe.
Nonglycine series ■— —■ μg proto, □— —□ /min/μg proto, × c/min/mg ^{14}C-hemin, ×——× c/min/mg F

as acetic acid and the free amino acids (KRUH and BORSOOK 1956 ERIKSEN 1957 NIZET 1957 MORELL, SAVOIE and LONDON 1958) It might be that the protoporphyrin present in young red cells differs from that of the true intermediate in its localization or its chemical properties or both. If preformed protoporphyrin lay on the pathway to heme, one would expect that the specific activity of heme should follow very closely that of free protoporphyrin, and cells suspended in 0.9 % NaCl/glycine (0.03 N) containing 2 μc methyl labelled acetate per 5 ml of cells, were incubated for 60 min in air. Samples were taken after 0, 7, 15, 30 and 60 min, and the amount of free protoporphyrin and the specific activities of free protoporphyrin and hemin were measured.

As seen from Fig 5 the amount of free protoporphyrin increases rapidly to approximately 5 times its zero value in 30 min, from then on the increase is only slight. The curves for the specific activities of free protoporphyrin and

hemin are not consistent with the assumption that preformed protoporphyrin is metabolically equal to the newly formed true intermediate, but indicate that more than one pool of protoporphyrin (or protoporphyrin precursor converted into protoporphyrin during the extraction procedure) is present in the system, and that only one of these is utilized for heme formation.

To test this possibility 2 bottles containing 40 ml of the standard reticulocyte suspension were incubated in air. To bottle 1 glycine was added in optimal concentration for protoporphyrin formation (ERIKSEN 1956). After 30 min incubation the cells and media were separated by centrifugation and the cells suspended in 2 volumes of 0.9 % NaCl plus ferric chloride in optimal concentration for heme formation (ERIKSEN 1956). Four series of bottles, two of which contained the cells preincubated with glycine and two containing the cells from the non glycine medium, were further incubated. As tracers were used carboxyl labelled acetate and ^{59}Fe as ferric chloride. The incubation was stopped after 0, 7.5, 15 and 30 min, and the amounts of free protoporphyrin and the specific activities of free protoporphyrin, hemin and hemin iron were measured.

As seen from Fig. 4 a, the amount of free protoporphyrin in the cells preincubated with glycine is considerably higher than in the non glycine cells. In both series the amount of preformed protoporphyrin keeps approximately constant throughout the second incubation. Thus if preformed protoporphyrin has been utilized for heme formation, an equivalent amount of protoporphyrin must have been formed.

The amount of heme formed as judged from the amount of ^{59}Fe incorporated, is equal in both series (Fig. 4 c), and the specific activity of free protoporphyrin from the non glycine cells is approximately twice that of free protoporphyrin from cells preincubated with glycine. If preformed protoporphyrin was equally well utilized for heme formation as the newly formed true intermediate, the C-activity of the hemin should vary in the same way as that of the free protoporphyrin. However, as seen from Fig. 4 b, the specific activities of the ^{14}C-labelled hemins are of the same order in both series and even slightly lower in the non glycine series. Thus the specific activity of free protoporphyrin could not be used as a measure of the specific activity of the true intermediate. It is further obvious that the true intermediate bypasses the main pool of protoporphyrin. Since, however, the specific activity of free protoporphyrin increases relatively rapid even in the glycine series and without any increase in the amount of free protoporphyrin, it means that a smaller part of the true intermediate is present and resynthesized during the second incubation.

Heme formation in hemolysates and whole cell systems under hypoxic conditions. The possibility that the only difference between the two pools is the localization within the heme forming system, would fit better with our earlier finding that added ^{14}C-labelled protoporphyrin is readily utilized for heme formation by rabbit reticulocytes in vitro (ERIKSEN 1956).

Table I Incorporation of ^{59}Fe and disappearance of preformed protoporphyrin in hemolysates incubated in air or a stream of N_2/CO

Time in min	Atmosphere	c/min/mg hemin Fe	per cent ^{59}Fe incorp.	Drop in proto in µg	Drop in proto in per cent of aerobic drop
30	air	1,784	100	23.4	100
30	N_2/CO_2	2,212	123.9	28.7	122.6

To test this possibility lysates of reticulocytes preincubated aerobically for 30 min in 0.9 % NaCl/glycine (0.03 M) were prepared by a method slightly different from that of Dresel and Falk (1954) and incubated in two series with ^{59}Fe as tracer. The only difference between our method and that of Dresel and Falk was that the cells were lysed at room temperature instead of at 0—4° C as used by these authors. A lysate prepared in this way has lost its ability to synthesize protoporphyrin and heme from acetate and glycine, but is still capable of forming heme from tetrapyrrolic intermediates (ERIKSEN, unpublished data).

Series 1 was incubated in air and series 2 was incubated in a stream of commercial N_2/CO (95/5). The amount of free protoporphyrin and the specific activity of hemin iron were measured at zero time and after 30 min of incubation. As seen from Table I, more heme has been formed in the N_2/CO series than in the aerobic series. In both series heme formation is followed by a drop in free protoporphyrin, and the drop is higher in the N_2/CO series than in the aerobic series. The incorporation of iron and the drop in free protoporphyrin in the N_2/CO series in per cent of the changes in the aerobic series are approximately identical indicating that the lost protoporphyrin has been utilized for heme formation. The results also support the above suggestion that the metabolic difference between the protoporphyrin fractions studied is due to the localization in the system used and not to chemical difference between protoporphyrin and the normal intermediate.

Since the N_2/CO atmosphere seems to favour the insertion of iron into protoporphyrin, the possibility existed that heme formation from preformed protoporphyrin might take place even in whole cells in a similar atmosphere, and experiments designed to test this possibility were undertaken. We soon found that when true anaerobiosis was obtained, no or negligible amounts of heme were formed, while with a gassing procedure identical with that used in the above experiments, iron was utilized for heme formation even better than in air. However, the drop in protoporphyrin was small and far from equivalent to the amount of heme formed. Thus the heme formed could not be due to utilization of preformed protoporphyrin, but must originate from the newly synthesized intermediate.

Table II Incorporation of ^{59}Fe and ^{14}C-acetate into heme in rabbit reticulocytes suspended in 2 vol. 0.9 % NaCl/lycine and incubated in air or stream of N_2/CO for 2 hours

Atmosphere	c/min/mg ^{14}C-hemin	c/min/mg hemin-^{59}F	sp. act. ^{59}Fe / sp. act. hemin- ^{14}C
air	100.0	18,460	184 6
N /CO_2	112.2	20,885	186.2

In Table II the results are given of an experiment designed to establish the possible origin of the newly formed heme.

Four series of cells suspended in 0 9 % NaCl/glycine were incubated for 2 hours. Series 1 and 2 were incubated in air and series 3 and 4 were incubated in N_2/CO_2. As tracers were used ^{14} C-labelled acetate and ^{59}Fe as ferric chloride.

As seen from the table, the amount of heme formed as judged from the amount of iron incorporated is approximately 10 per cent higher in the N_2/CO series as compared to the aerobic series, in good agreement with the findings in the experiments with hemolysates. However the C incorporation has increased to the same degree, and the ratio of ^{14}C-hemin/hemin-^{59}Fe is identical in both series. Thus the heme formed must have the same origin in both series.

The N_2/CO mixture was found to contain less than 3 per cent oxygen, and the incubation bottles were flushed with the two gas mixtures for 60 min prior to the introduction of tracer thus the condition in the N_2/CO series must be highly hypoxic, and even under these conditions the heme forming system seems to prefer the newly formed intermediate corresponding to protoporphyrin for preformed protoporphyrin.

Discussion

As shown in Fig 1 the amount of both free protoporphyrin and coproporphyrin are closely related to the number of reticulocytes both during the development and the repair of a heavy anemia. However the form of the curves is compatible with the assumption that the main part of the free porphyrins is present in cells of an intermediate maturation state, which would explain the findings of WATSON and CLARKE (1937) WATSON et al. (1944) and SCHWARTZ and WIKOFF (1952)

The rap d drop in free porphyrin from the 7th to the 9th day might be due either to utilization of the porphyrins for heme formation or disposal of them by other routes. Since as shown in Fig 3 and 4 preformed protoporphyrin is only poorly utilized for heme formation in vitro although heme formation from simple metabolites such as acetic acid goes on with a considerable speed, and

since preformed protoporphyrin is incapable to dilute the incorporation of ^{14}C from added acetic acid, it seems reasonable to assume that the true intermediate corresponding to protoporphyrin differs from preformed protoporphyrin either in its site of localization or in its chemical properties or both.

Added protoporphyrin is readily utilized for heme formation both in whole cell systems (ERIKSEN 1956) and cell free systems (KEVORK et al 1956, GOLDBERG et al. 1956, MINAKAMI et al. 1958) thus if the true intermediate does differ chemically from protoporphyrin the latter must be easily converted into the former even in such extremely simple systems as the 50 per cent ammonium sulfate fraction of cholate extracts of the insoluble part of duck erythrocytes described by KAGAWA, MINAKAMI and YONEYAMA (1958)

The observation that higher amounts of heme are formed when the incubation is performed in a stream of N_2/CO (Table I and II and GRANICK, 1954) and the findings of several authors that reducing substances such as cysteine, glutathione and ascorbic acid increase heme formation from protoporphyrin (MINAKAMI 1958 MINAKAMI et al. 1958 GOLDBERG 1959 NISHIDA and LABBE 1959) might suggest that some reduction of the protoporphyrin molecule is necessary for the utilization in heme formation. However as shown by KAGAWA et al. (1958) these compounds seem to play no role as reductants neither for protoporphyrin nor for iron, but to protect some easily oxidizable group or compound in the heme forming system. It has also been shown by NISHIDA and LABBE (1959) that protoporphyrin is more readily utilized for heme formation than protoporphyrinogen in cell free systems.

Thus, although the question can not be said to be settled, the evidence is in favour of the assumption that protoporphyrin in contrast to other porphyrins is a true intermediate in heme formation, nd that the non utilization of preformed protoporphyrin is due to the localization of excess formed protoporphyrin in the system studied, an assumption which is favoured by our own finding (Table I) that in disrupted cells preformed protoporphyrin is readily utilized for heme formation.

Since the cells used in the above in vitro experiments most pr bably are a population of cells of different maturation and thus with different bility to form porphyrins and heme, t becomes difficult to decide whether this compartmentation of protoporphyrin can occur within a single cell or is due to the distribution of protoporphyrin between cells of different maturation. However DRESEL and FALK's observation that protoporphyrin added to hemolysates does not dilute the activity incorporated into heme from different tracers added when the lysates are prepared in such a way that the heme forming enzymes are still located in the particulate matter (DRESEL and FALK 1956) indicates that this phenomenon does occur even u non cellular systems, and preliminary results dealt with elsewhere (ERIKSEN and ERIKSEN 1960) indicate that the formation and utilization of protoporphyrin for heme formation is connected with the particulat matter of the reticulocytes, and that excess formed proto-

porphyrin diffuses out into the soluble part of cytoplasm and becomes unavailable for heme formation.

The fate of this protoporphyrin fraction is so far unknown, but preliminary investigations suggest that the ultimate fate of excess formed protoporphyrin is the breakdown to bile pigment or related compounds, and the possibility exists that the socalled "early" bile pigment fraction formed in direct relation to hemoglobin biosynthesis in mammals (LONDON et al. 1950 GRAY NEUBERGER and SNEATH 1950) does origin from this protoporphyrin fraction, and experiments designed to test this possibility are in progress at our institute.

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From The Institut for Medical Biochemistry and Physiology Department of Physiology
University of Oslo, Norway

The Effect of Cobalt Ions on the Biosynthesis of Hemoglobin by Rabbit Reticulocytes in Vitro

By

LEIF ERIKSEN NORA ERIKSEN and SYNNØVE HAAVALDSEN

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Abstract

ERIKSEN L. N ERIKSEN and S. HAAVALDSEN *The effect of cobalt ions on the biosynthesis of hemoglobin by rabbit reticulocytes in vitro.* Acta physiol. scand. 1961 *53* 300—307 — The effect of cobalt ions on the biosynthesis of hemoglobin by rabbit reticulocytes in vitro has been studied. It is shown that cobalt ions in concentrations of approximately 10[illegible] M almost completely inhibit the formation of heme, while the formation of globin is unaffected or slightly stimulated. The inhibiting effect on heme formation is found to be due to inhibition of the formation of the tetrapyrrolic intermediates in heme formation, while the conversion of the 8 carboxylic intermediate by stepwise decarboxylation and finally dehydrogenation and iron corporation is unaffected. The implications of the above findings for the understanding of the effect of cobalt ions on the erythropoiesis are discussed, and it is suggested that the polycythemia induced by cobalt ions may be due to a toxic effect of the metal on hemoglobin formation with secondary stimulation of red cell formation. Th possibility that cobalt may exert directly stimulating effect on the formation of erythropoietine is not, however excluded.

It has long been known that cobalt salts when administered in small doses to man or experimental animals, cause development of a polycythemia. How this effect is exerted is not known although a series of suggestions have been put forth (for details see GRANT and ROOT 1952)

The finding of MORELL, SAVOIE and LONDON (1958) that cobalt ions in low concentrations do inhibit the incorporation of C-labelled glycine into the heme group of hemoglobin in red bone marrow cells in vitro while the incorporation of glycine into globin was unaffected or slightly stimulated *is of great*

interest and might give some clue to the understanding of the effect of cobalt on the erythropoiesis. If as assumed by MORELL *et al.* the decreased incorporation of added glycine into heme actually represents a decreased formation of the heme group the finding of GOLDWASSER *et al* (1957) that the erythropoietine titer of plasma from cobalt polycythemic rats is increased in relation to normal rat plasma, might be due to the formation of red cells poor in hemoglobin with secondary increase in the erythropoietine titer Since however MORELL *et al.* used only C-labelled glycine as marker it could not be definitely stated whether the in vitro effect of cobalt observed represented an inhibition of heme formation or whether it only meant that the utilization of added glycine for heme formation was inhibited, while heme formation as such went on with unaltered speed. We therefore undertook the following experiments to get more definite information on the effect of cobalt on hemoglobin biosynthesis

Material and methods

Reticulocyte rich blood was obtained from rabbits made anemic by daily injections of 25 mg phenylhydrazine for 5 days and killed on the 7th day

The incubation technique and the methods used have been described in details elsewhere (ERIKSEN 1955 a, b, 1956)

As tracers were used glycine labelled in the methyl group and acetate labelled in the carboxyl group with ^{14}C, and ferric chloride labelled with ^{59}Fe. The specific activities of the tracers were glycine 2-^{14}C 2 mc/mmole, acetate 1-^{14}C 1 mc/mmole and $FeCl_3$ 50 mc Fe/g Fe. All the isotopes have been obtained from Amersham, England.

Experimental and results

1) The effect of Co^{++} on the incorporation of glycine-2-^{14}C into heme and globin

Two series of bottles were incubated in air for 1 hour at 37° C. Each bottle contained 5 ml cells suspended in 2 volumes of 0.9 % NaCl + 2 μc ^{14}C-glycine + $FeCl_3$ (90 μg Fe) To series 2 cobalt was added as cobaltous chloride to a final concentration of 3×10^{-3} M.

At the end of the incubation the cells were washed twice with 2 volumes of 0.9 % NaCl and lyzed by freezing and thawing, and finally diluted with 2 volumes of distilled water The lysates were spun in the Spinco for 90 min at 40,000 r. p. m. and the supernatant used for hemin and globin preparation

Hemin and globin were plated and counted in the usual way (ERIKSEN 1956) and the activity calculated in infinitely thick layers.

As seen from Table I Co^{++} almost completely inhibits the incorporation of glycine into heme, while the incorporation into globin is slightly stimulated in complete accordance with the results of MORELL *et al.* (1958)

2) The effect of Co^{++} on the incorporation of ^{59}Fe and ^{14}C-acetate into heme

Experiments similar to those described above were performed. However as tracers were used carboxyl labelled acetate and ^{59}Fe labelled ferric chloride.

Table I. The effect of cobalt on the incorporation of C-labelled glycine into heme and globin by rabbit reticulocytes in vitro

Time in min	Medium	c/min infinitely thick	
		Hemin	Globin
60	Co^{++}	1	18.6
	control	386.5	19.2

Table II. The incorporation of ^{59}Fe *and* ^{14}C*-labelled acetate into heme by rabbit reticulocytes in vitro with and without cobalt added*

Time in min	Medium	c/min/mg	
		C-Hemin	Hemin-Fe
60	Co^{++}	90.7	147.1
	control	533.7	2,247.1

Table III. The effect of cobalt on the formation of free protoporphyrin by rabbit reticulocytes in vitro

Time in min	µg protoporphyrin	
	Co^{++}	control
0	9.1	9.1
60	6.5	45.2

As seen from Table II Co^{++} almost completely inhibits the incorporation of both iron and acetate into heme, thus there is very good evidence for the assumption that the decreased incorporation of glycine into heme described by MORELL *et al.* and in the above experiment is due to a true inhibition of heme formation.

5) The effect of Co on the formation of free protoporphyrin

Cells suspended in 0.9 % NaCl/glycine (0.03 M) were incubated in air and the amount of free protoporphyrin was measured at zero time and after *1 hour* of incubation with and without cobalt added. As seen from Table III the amount of free protoporphyrin is slightly lower after 1 hour of incubation than at zero time in the cobalt series while in the control the amount of protoporphyrin has increased to a value of approximately 5 times the zero value. Thus the formation of protoporphyrin is strongly inhibited by cobalt ions.

Table IV. The effect of cobalt on heme formation by rabbit reticulocytes in vitro when ^{14}C-labelled protoporphyrin has been accumulated in the cells prior to the addition of cobalt

Time min	Series	μg proto	c/min/μg proto	c/min/mg ^{14}C-Hemin	Hemin-F
0	Cells preinc. in 0.9 % NaCl glycine (0.03 M) + 1 ^{14}C-acetate	11.2	187.1	180.5	—
	Controls				
30	I $FeCl_3$	15.9	134.2	275.7	
	III $^{59}FeCl_3$				4,500.1
	Co^{++} 3×10^{-3} M				
	II $FeCl_3$	9.9	173.4	179.4	
	IV $^{59}FeCl_3$				150.0

4) The effect of Co^{++} on heme formation when free protoporphyrin or other possible tetrapyrrolic intermediates in heme formation have been accumulated in the cells prior to the addition of cobaltous chloride

The slight drop in the amount of free protoporphyrin in the cobalt series in the previous experiment, might indicate that some of the preformed protoporphyrin had been utilized for heme formation. To test this possibility the following experiment was performed.

Cells suspended in 0.9 % NaCl/glycine (0.03 M) with ^{14}C-labelled acetate as tracer were incubated for 30 min in air. Under these conditions the only possible tetrapyrrolic intermediate in heme formation labelled with ^{14}C and present in measurable amounts in the cells after the incubation would be protoporphyrin (ERIKSEN 1955 a, 1956; ERIKSEN, ERIKSEN and HAAVALDSEN 1960).

At the end of the incubation the cells were centrifuged down, washed twice with 2 volumes of 0.9 % NaCl and resuspended in 0.9 % NaCl/glycine, and 4 series were incubated further in air for 30 min. To series I and II inactive ferric chloride was added and to series III and IV ferric chloride labelled with ^{59}Fe. The final concentration of iron in the incubation bottles was 600 μg % Fe. To series II and IV cobaltous chloride was added to a final concentration of 3×10^{-3} M.

The amount of free protoporphyrin and the specific activities of free protoporphyrin and hemin were measured before and after the second incubation.

As seen from Table IV, no or negligible amounts of heme have been formed in the cobalt series, while in the control a substantial amount of heme has been formed as judged both from the amount of ^{59}F incorporated and the increase in the specific activity of ^{14}C labelled hemin.

Table V. The effect of cobalt on heme formation by rabbit reticulocytes in vitro when ^{14}C-labelled intermediates corresponding to uro- and coproporphyrin have been accumulated in the cells prior to addition of cobalt

Time in min	Series	Atmosphere	Type of porphyrins found			cts/mg Hemin
			uro	copro	proto	
0	Cells preinc. in plasma/glycine (0.03 M) + $1\text{-}^{14}C$-acetate.	N_2/CO	2+	4+	2+	104.5
30	Control	air	—	trace	6+	133.2
	Co^{++} 5×10^{-3} M	air	—	trace	6+	139.5

As shown in the preceding paper (ERIKSEN 1961) preformed protoporphyrin is only poorly utilized for heme formation in cell systems of the type used in these experiments. Thus, even though cobalt may inhibit the formation of heme from iron and the true intermediate corresponding to protoporphyrin, it must, especially since the drop in the specific activity of free protoporphyrin in the cobalt series is lower than in the control, have an inhibitory effect at some earlier step in the sequence of events leading to heme. Information on the possible step in heme formation inhibited by cobalt ions was sought in the following way.

Cells suspended in 2 volumes of plasma/glycine (0.03 M) + carboxyl labelled acetate were incubated for 60 min in a stream of N_2/CO containing approximately 3 % oxygen. Under these conditions intermediate tetrapyrroles having from 8—2 carboxyl groups will accumulate in the cells (ERIKSEN 1955 a, 1956, ERIKSEN *et al.* 1960). At the end of the incubation cells and plasma were separated by centrifugation and the cells washed twice with 0.9 % NaCl. The main part of the cells were suspended in 2 volumes of 0.9 % NaCl/glycine (0.03 M) containing iron as ferric chloride in a concentration of 600 µg Fe per cent. The suspension was divided into 2 series and further incubated in air for 30 min. To one of the series cobalt was added as cobaltous chloride to a final concentration of 5×10^{-3} M.

The specific activity of hemin was measured before and after the second incubation. The total amount of the free tetrapyrrolic intermediates was not measured. However, all the intermediate tetrapyrroles were extracted with ethylacetate/glacial acetic acid (4/1) and converted into porphyrins as described elsewhere (ERIKSEN 1956). The porphyrins were adsorbed to small talc columns, washed and eluted with 10 % NH_3/acetone (3/7), applied to paper and chromatographed according to ERIKSEN (1953).

As seen from Table V, tetrapyrroles giving rise to porphyrins having from 8—2 carboxylic groups were present in the cells prior to the second incubation, and the main porphyrin behaved as coproporphyrin. After the second incuba-

Table VI The effect of cobalt on the incorporation of ^{59}Fe into heme by rabbit reticulocytes in vitro when intermediates corresponding to uro- and coproporphyrin have been accumulated in the cells prior to the addition of cobalt

Time in min	Series	Atmosphere	Type of porphyrins found: uro	copro	proto	/min/mg F
0	Cells preinc. in plasma/glycine (0.03 M)	N_2/CO	2 +	4 +	2 +	
30	Control	air	—	trace	6 +	3,332.1
	Co^{++} 3×10^{-4} M	air	—	trace	6 +	3,328.0

tion the porphyrin pattern has changed completely and in the same way in both series. The main porphyrin is now behaving as protoporphyrin, coproporphyrin is present in negligible amounts only and no trace of porphyrins having more than four carboxyl groups are found. It is further seen from the table that the specific activities of the hemins have increased approximately 30 % in both series.

Since neither the porphyrin pattern nor the amount of the newly formed heme are different in the cobalt series from that of the control, it becomes evident that cobalt has no effect neither on the conversion of the tetrapyrrolic intermediates into the true heme precursor nor on the insertion of iron into this compound.

To exclude the possibility that the ^{14}C activity found in hemin should be accidental and due to some unspecific contamination of the hemin isolated, the experiment was repeated, and as a control was used 2 series in which ^{59}Fe was used as tracer. The results were completely identical with those cited, and as shown in Table VI, the amount of heme formed during the second incubation as judged from the amount of ^{59}Fe incorporated is the same whether cobalt has been added or not.

We have previously shown that the heme formed is true protopheme (ERIKSEN 1955 a, 1956) thus there is no reason to believe that the ^{14}C and ^{59}Fe activities of the hemins should be due to admixture of trace amounts of uroheme or coproheme, an assumption which in itself does not seem very probable on the basis of the above experiments.

Discussion

Our findings are in complete accordance with those of MORELL *et al.* (1958) and show that the inhibition of ^{14}C-glycine incorporation into the heme group by cobalt form described by these authors is due to the inhibition of heme formation. Our data also show that the inhibitory effect of cobalt on heme formation is due to a block in the formation of the tetrapyrrolic intermediates in heme formation at some step prior to uroporphyrinogen, while the conversion

of this latter into the copro- and proto-type intermediates and the final insertion of iron into the latter is unaffected.

The results cited in Table IV and V also support our finding in the preceding paper (ERIKSEN 1961) that preformed protoporphyrin is not or only poorly utilized for heme formation in hemoglobin forming systems of the type described. The above results also strongly support our previous finding that protoporphyrin and globin are formed by completely independent processes and can be taken as support for the assumption that globin acts as an acceptor for the normal protoporphyrin type intermediate in heme formation or for heme itself (ERIKSEN 1955 a, 1956 1957 1960) The latter possibility has been discussed in details elsewhere (ERIKSEN 1960) and the reader is referred to this paper

It is obviously too early to extend the above in vitro findings to the situation in whole animals. It is, however interesting that DAVIS and FIELDS (1958) found that the red cells produced in cobalt induced polycythemia in man were hypochromic indicating that the formation of new red cells was less affected than the formation of the hemoglobin molecule.

SAIKKONEN (1959) found that cobalt administration to rat caused a significant increase in coproporphyrin excretion parallel with a small but significant drop in the hemoglobin concentration in peripheral blood. After 10—14 days a true polycythemia developed. A series of other poisonous effects were also described, and SAIKKONEN pointed upon the similarity of the action of cobalt to heavy metal poisoning

The findings of DAVIS and FIELDS (1958) and SAIKKONEN (1959) when taken together with our own in vitro findings, could be taken as support for the assumption that the increased erythropoietine titer in cobalt polycythemic rats described by GOLDWASSER *et al.* (1957) is secondary to the formation of red cells poor in hemoglobin They do not, however exclude the possibility that cobalt apart from the toxic effect described above do exert a directly stimulating effect on the erythropoietine forming tissues, and further experiments are needed

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From The Institute for Medical Biochemistry and Physiology Department of Physiology, University of Oslo, Norway

The Formation of Coproporphyrin III and I by Rabbit Reticulocytes Under Hypoxic Conditions in Vitro

By

Leif Eriksen Nora Eriksen and Synnøve Haavaldsen

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Abstract

Eriksen L., N Eriksen and S. Haavaldsen. *The formation of coproporphyrin III and I by rabbit reticulocytes under hypoxic conditions in vitro.* Acta physiol. scand. 1961 53. 308—314. — It has been shown that tetrapyrroles having from 4 to 8 carboxylic groups of both the III and I isomer series are formed by rabbit reticulocytes in vitro under hypoxic conditions. The tetrapyrroles of the III series are rapidly converted into protoporphyrin under aerobic conditions, while the tetrapyrroles of the I series are converted into coproporphyrinogen I. The implications of these findings have been discussed and it is suggested that an 8-carboxylated openchained tetrapyrrolic intermediate of the I series is normally formed by deaminase, the so-called porphobilinogenase, and that this tetrapyrrole is condensed spontaneously into uroporphyrinogen I if this condensation is not hindered by second enzyme the so-called isomerase. The possible effect of the latter is discussed and a scheme for the formation of ringformed tetrapyrroles of both the III and I series has been suggested. It is further suggested that the formation of ringformed tetrapyrroles of the I series is due to inhibition or blockage of the isomerase with spontaneous condensation into uroporphyrinogen I of the openchained tetrapyrrole of the I series formed by the deaminase uroporphyrinogen I being rapidly converted by the decarboxylases present into coproporphyrinogen I. The accumulation of the latter when the atmosphere is changed into air shows that the enzymes responsible for the conversion of coproporphyrinogen into protoporphyrin are highly specific and can utilize only coproporphyrinogen III and explains why porphyrins having less than 4 carboxylic groups have been found in nature only as isomers of the III series.

In a series of previous papers we have shown that tetrapyrrolic intermediates in heme formation tend to accumulate in rabbit reticulocytes under hypoxic conditions in vitro (Eriksen 1955, 1956, Eriksen, Eriksen and Haavaldsen 1957).

Since under physiological conditions both man and rabbit excrete small amounts of at least three different coproporphyrin precursors (Eriksen, Eriksen and Haavaldsen 1961 a), giving rise to the coproporphyrin isomers III, I and trace amounts of a porphyrin behaving as the II isomer of coproporphyrin (Eriksen 1953 a, Eriksen et al. 1961 a, b), the possibility did exist that these coproporphyrin precursors were formed during the biosynthesis of heme. To test this possibility a study of the isomer pattern of coproporphyrin formed from rabbit reticulocytes incubated in plasma glycine under highly hypoxic conditions was undertaken.

Experimental and Results

The technique used for the preparation of blood rich in reticulocytes from rabbits has been described in detail elsewhere (Eriksen 1955).

The cells were suspended in 2 volumes of plasma glycine (0.022 M) and incubated in an atmosphere of N_2/CO containing less than 3 per cent oxygen.

The accumulated tetrapyrroles were extracted with ethyl acetate-glacial acetic acid (4:1), converted into porphyrins by extraction with 3 N HCl and (Eriksen 1956) and separated by means of paper chromatography (Eriksen 1953). The zone corresponding to coproporphyrin was cut out, eluted with 3 N HCl and, adsorbed to small talc columns, eluted with 10 per cent NH_4 acetate (pH 3.7) and finally the purified coproporphyrin fraction was applied to paper and chromatographed in our coproporphyrin isomer separating system (Eriksen, 1958 b).

As seen from Fig. 1, only the III isomer of coproporphyrin is present in measurable amounts. A slight tailing might indicate the presence of trace amounts of the I isomer. However, the isomer pattern is completely different from that found in the urine of normal man and rabbit in which, although the III isomer is quantitatively dominating, a considerable amount of the I isomer is also present together with trace amounts of a possible II isomer (Eriksen et al. 1961 a, b).

As mentioned above, the slight tailing observed might be due to the presence of trace amounts of the I isomer. To test this possibility the following experiments were undertaken.

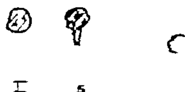


Fig. 1. Isomer chromatogram of the coproporphyrin fraction obtained from reticulocytes incubated in plasma glycine under hypoxic conditions. S = sample, III = coproporphyrin III, I = coproporphyrin I.

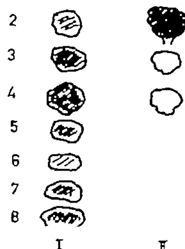


Fig. 2. Chromatogram of porphyrins found in rabbit reticulocytes after preincubation in plasma/glycine under hypoxic conditions (I) and reincubation in NaCl, glycine in air (II). System lutidine/H_2O 5/3 + NH_3, unsaturated.

Rabbit reticulocytes incubated as described above were suspended in 2 volumes of saline/glycine (0.032 M) and incubated in air for 30 min, and the whole incubate was worked up on porphyrins in the usual way.

Under these conditions all the tetrapyrroles of the III series would be converted into protoporphyrin IX (ERIKSEN 1955, 1956, ERIKSEN et al. 1960), while the I isomers would stop at the coproporphyrinogen level (ERIKSEN, HAAVALDSEN and ERIKSEN 1960). Thus if tetrapyrroles having 4 or more carboxylic groups were present prior to the aerobic incubation, all the I isomers should be trapped as coproporphyrinogen I after the aerobic incubation.

As seen from Fig. 2, approximately all the porphyrin present after aerobic incubation is present as a dicarboxylic porphyrin which has been identified as protoporphyrin.

Small amounts of porphyrins behaving as 5 and 4 carboxylic are also present. The amount of these latter porphyrins was very small and was difficult to handle even with the micro technique described elsewhere (ERIKSEN et al. 1961 b). To get information about the isomeric nature of the coproporphyrin without losing appreciable amounts of porphyrin, a chromatogram was turned 90° and chromatographed in our coproporphyrin isomer separating system (ERIKSEN 1958 b). As seen from Fig. 3, the coproporphyrin spot has split into two distinct spots corresponding to the III and I isomers. The amount of porphyrin as judged from the colour and fluorescence of the spots is approximately equal. Thus the ratio of I/III of coproporphyrin has changed from approximately zero to 1. The presence of some III isomer after reincubation in air most probably is due to oxidation of the true intermediate during the aerobic incubation.

To exclude the possibility that the I isomer had been formed during the aerobic incubation, the experiment was repeated. However, this time cobaltous



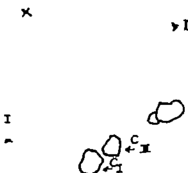


Fig. 3. Twoways chromatogram of porphyrins found in reticulocytes preincubated in plasma/glycine under hypoxic conditions and reincubated in NaCl/glycine in air. System I, lutidine/H_2O 5/3 + NH_3 unsaturated, system II, lutidine/H_2O 5/2 + NH_3 saturated.

Fig. 4. Twoways chromatogram of porphyrins found in reticulocytes preincubated in plasma/glycine under hypoxic conditions and reincubated in NaCl/glycine in air with Co^{++} added. System I, lutidine/H_2O 5/3 + NH_3 unsaturated, system II lutidine/H_2O + NH_3 saturated.

chloride was added to the aerobic incubation bottles to a final concentration of 3×10^{-4} M. Cobalt ions of this concentration inhibit almost completely the formation of the tetrapyrrolic intermediates in heme formation, while it has no effect on the conversion of these intermediates into protoporphyrin and heme (ERIKSEN 1960, ERIKSEN *et al.* 1961 c). Thus, if tetrapyrroles of the I series are present after the aerobic incubation, these must origin from tetrapyrroles formed during the hypoxic incubation and having 4 or more carboxylic groups.

As seen from Fig. 4 the isomer pattern of coproporphyrin is identical with that obtained in the above experiment. Thus there is very good reason to assume that tetrapyrroles of both the III and I series have been formed under hypoxic conditions. The amount of the tetrapyrroles of the I series is extremely small as compared to that of the III series.

Discussion

As shown by BOGORAD (1958) two different enzymes seem to be needed for the conversion of porphobilinogen into uroporphyrinogen III a deaminase (porphobilinogenase) giving rise to a polypyrrole with a sidechain sequence corresponding to the I series, and an "isomerase directing the final condensation in such a way that one (or three) of the pyrroles is rotated to give a sidechain sequence of the III series prior to the condensation into uroporphyrinogen.

Uroporphyrinogen I can not be utilized as a substrate for the isomerase (MAUZERALL and GRANICK 1958) thus it is reasonable to assume that the substrate is an openchained tetrapyrrole with a sidechain sequence corresponding to the I series, and which, if the isomerase is destroyed by heat (GRANICK and MAUZERALL 1958, BOOIJ and RIMINGTON 1957) or limited by accumulation of product as in the above experiments, would condense chemically into uroporphyrinogen I

Since the uroporphyrinogen decarboxylase responsible for the conversion of uroporphyrinogen into coproporphyrinogen does not distinguish between the different isomers (MAUZERALL and GRANICK 1958) any uroporphyrinogen I formed would be expected to be converted into coproporphyrinogen I when the block in protoporphyrin formation due to hypoxia is released during the aerobic incubation, similar to what has been found above.

Our findings may be explained according to the following scheme for protoporphyrin formation from porphobilinogen (Fig 5)

According to this scheme the isomerase acts by blocking the free α-carbon in ring A and loosening the linkage between the other α-carbon and the methylene bridge carbon, allowing the A ring to rotate and the methylene carbon in the D ring to link to the carbon atom set free by the isomerase, thus giving rise to a tetrapyrrole of the III series which finally can condense into uroporphyrinogen III If the isomerase is destroyed or limited the openchained tetrapyrrole would be expected to condense almost spontaneously to uroporphyrinogen I

The above scheme is supported by the finding of BOGORAD (1958) that the deaminase isomerase system under certain conditions can be split into two subfractions, fraction I being capable to form uroporphyrinogen I with porphobilinogen as a substrate while fraction II can not use porphobilinogen as a substrate at all However addition of fraction II to fraction I gives rise to the formation of uroporphyrinogen III when incubated with porphobilinogen. The deaminase thus seems to be the enzyme responsible for the condensation of the porphobilinogen molecules into the tetrapyrrole, which, if not directed by the isomerase, will condense into uroporphyrinogen I

Since the isomerization and condensation takes place without the release of formaldehyd or any other one carbon compound (LOCKWOOD and BENSON 1960) it means that the isomerization that is the migration of one bridge carbon atom from one α-position to another via the N atom, most probably takes place after the formation of the immediate tetrapyrrolic precursor to uroporphyrino-

Fig. 5

4 Porphobilinogen

Deaminase
— 4 NH

↓ Isomerase

Nonenzymic

Uroporphyrinogen I

↓ Dec.

Coproporphyrinogen I

Uroporphyrinogen III

↓ Dec.

Coproporphyrinogen III $\xrightarrow[\text{Dehydr}]{\text{Dec.}}$ Protoporphyrin IX ⟶ Heme

gen, and which, if the isomerase is not present, will condense spontaneously into uroporphyrinogen I. The possibility that the condensation takes place prior to the isomerization seems to be definitely excluded by the finding of GRANICK and MAUZERALL (1958) that uroporphyrinogen I can not serve as a substrate for the isomerase.

The mechanism suggested by BULLOCK *et al.* (1958) for the chemical formation of coproporphyrin III, assuming a migration of the methylene group from the D ring to the free α-carbon of the A ring, thus changing the sidechain sequence with final addition of the B and C rings to give the III isomer, can not explain the formation of the I isomer when the isomerase is destroyed or inhibited, since the tetrapyrrole formed would have a sidechain sequence identical to that of the III series, and one (or three) of the pyrrole rings had to be rotated prior to condensation to give uroporphyrinogen I, a possibility which does not seem very likely.

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From the Department of Pharmacology Karolinska Institutet, Stockholm, Sweden

Evidence for Energy-Requiring Processes in Histamine Release and Mast Cell Degranulation in Rat Tissues Induced by Compound 48/80

By

BERTIL DIAMANT and BÖRJE UVNÄS

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Abstract

DIAMANT B. and B. UVNÄS. *Evidence for energy-requiring processes in histamine release and mast cell degranulation in rat tissues induced by Compound 48/80* Acta physiol. scand. 1961 53. 315—329 — Histamine release from rat lung tissue and degranulation of rat mesentery mast cells were produced *in vitro* by Compound 48/80 Both processes were shown to be blocked by anoxia and by inhibitors of oxidative phosphorylation. Preincubation with glucose counteracted this inhibition. The results indicate that histamine release and mast cell degranulation require energy which can deriv from glucose metabolism.

Most authors agree that anaphylactic histamine release from sensitized guinea-pig lung tissue *in vitro* requires oxygen, since anoxic conditions and metabolic inhibitors, such as dinitrophenol, azide and cyanid (according to some reports, this occurs only in rather high concentrations) depress such release (PARROT 1942, MONGAR and SCHILD 1957 MOUSSATCHÉ and PROVOUST DANON 1958, CHAKRAVARTY 1960) It was stated, however that antigen-induced histamine release from sensitized rat lung tissue *in vitro* was not influenced by anoxia (CHAKRAVARTY 1959) On the other hand, degranulation of mast cells from sensitized rats was blocked by metabolic inhibitors, including those which are assumed to uncouple oxidative phosphorylation and to block respiratory enzymes (HÖGBERG and UVNÄS 1960 MOTA and ISHII 1960)

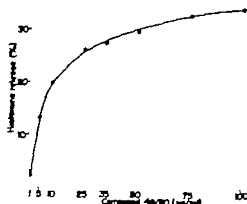


Fig. 1 Dose-response curve for Compound 48/80-induced histamine release from rat lung tissue under oxygen in presence of glucose (5.6 mM) Histamine release computed in per cent of total histamine content. Spontaneous release deducted from all values.

As opposed to these results for sensitized rat tissues, degranulation of non-sensitized rat mast cells, caused by extracts from Ascaris suis and Cyanea capillata, is blocked by metabolic inhibitors (UVNÄS *et al* 1960, UVNÄS 1960) and the histamine release from non-sensitized rat lung tissue *in vitro* due to Ascaris extracts, is blocked by anoxic conditions (DIAMANT 1960 1961)

Views diverge very considerably regarding the operative mechanism of the polymer amine, Compound 48/80 Its histamine releasing action is regarded as being independent of oxygen (rat lung CHAKRAVARTY 1959 cat paw CHAKRAVARTY HÖGBERG and UVNÄS 1959) In fact, histamine release from guinea pig lung tissue, due to Compound 48/80 was stated to be enhanced by oxygen lack and by metabolic inhibitors (MONGAR and SCHILD 1957) Recently however it was reported that metabolic inhibitors and anoxia reduce the histamine release from cat skin (WESTERHOLM 1960 1961) and rat tissues (MOTA and ISHII 1960)

Degranulation of rat mast cells induced by Compound 48/80 was reported to be blocked by metabolic inhibitors (JUNQUEIRA and BEIGUELMAN 1955, HÖGBERG and UVNÄS 1957 1960 MOTA and ISHII 1960) On the other hand, the incubation of rat mast cells under nitrogen did not abolish degranulation (HÖGBERG and UVNÄS 1960)

Since it was shown (DIAMANT 1960) that the presence of glucose enhanced the histamine release, induced by extracts of Ascaris suis, from rat lung tissue and since similar observations were reported regarding the histamine release induced by Compound 48/80 from cat skin *in vitro* (WESTERHOLM 1960) it was suggested that the divergent results of earlier investigations on the effect of Compound 48/80 could in part, be attributed to the presence of glucose Consequently in this report, we have re-investigated with regard to the effect of glucose, the influence of metabolic inhibitors and anoxia on the histamine release and the mast cell degranulation induced by Compound 48/80 in the rat.

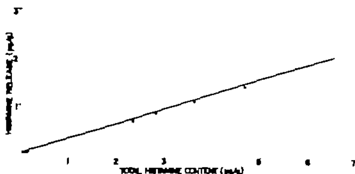


Fig. 2. Histamine release from rat lung tissue under nitrogen in presence of glucose (5.6 mM) induced by Compound 48/80 (35 µg/ml) as related to the total histamine content in 23 different experiments. All values given as µg histamine base per g of wet lung tissue. Spontaneous release deducted from all values.

In previous communications from this laboratory (Högberg and Uvnäs 1960) evidence has been presented to support the view that the mode of action of Compound 48/80 is, in principle, the same as that of antigen and histamine releasers extracted from natural sources, such as Ascaris and Cyanea. The present investigation will provide further evidence to strengthen this view

Methods and Materials

Incubation medium

In all experiments, we used, as incubation medium, a solution containing NaCl (154 mM) KCl (2.7 mM) and $CaCl_2$ (anhydrous, 0.9 mM) buffered with Sörensen phosphate buffer (67 mM) 10 per cent v/v The pH of the medium was between 7.0 and 7.2 in the experiments with isolated rat lung tissue and 6.9—7.4 in those on the degranulation of rat mesentery

Histamine release from isolated rat lung tissue

The technique used was similar to that described by Diamant (1961) but with slight modifications The lungs were excised from male and female rats, weighing 250—400 g In each experiment 3—6 animals were used. After removal of visible bronchi, all lung lobes were pooled. They were then cut into smaller pieces (1—2 mg) After careful washing with the incubation medium, the lung tissue was blotted dry It was subsequently divided into smaller parts by weighing The weight of each sample was usually between 300—600 mg (in no case less than 400 mg) Thus, a solution containing no glucose was used for the entire preparation of the lung samples. This could be accomplished without affecting the sensitivity of the tissue to Compound 48/80, since preliminary results had shown that there was no difference in the histamine release in a medium containing glucose, irrespective of whether or not the lung tissue had been treated, before incubation, with a solution containing glucose (5.6 mM)

The lung samples were incubated and shaken in stoppered Erlenmeyer flasks in a Warburg apparatus at 37 C. Unless otherwise indicated, glucose or other carbohydrates, when tested, were present in the Erlenmeyer flasks from the beginning of the incubation.

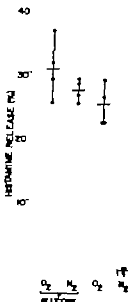


Fig. 3. The effect of glucose (5.6 mM) on histamine release induced by Compound 48/80 (35 μg/ml) from rat lung tissue under the influence of oxygen and nitrogen. Filled and open circles represent the individual values of double tests from t different experiments. All release values computed in per cent of total histamine content. Spontaneous release deducted from all values.

Oxygen or nitrogen gas was bubbled through, via syringe needles, during the first 15 min of incubation. Compound 48/80 was then added (with a final concentration of 35 μg/ml, except where otherwise stated) and, at the same time, the syringe needles were withdrawn above the surface of the solutions. Then, 20 min after the addition of Compound 48/80 the incubation fluids were withdrawn. In the text, this incubation procedure is referred to in an abbreviated form, i. e. 15 min glucose N_2 + 20 min Compound 48/80 N_2. Similar abbreviations are also used for other incubation procedures of lung tissue as well as of isolated mesentery. The remaining histamine in each lung tissue sample was extracted by heating to 100° C on a water bath for 10 min. Histamine was assayed on atropinized guinea pig ileum. The contractions were blocked by antihistamines, showing that the contractions were due to histamine. All values given are corrected for the spontaneous histamine release.

When dinitrophenol (DNP) was studied, the lung samples were preincubated with DNP for 15 min. During this time no gases were bubbled through the incubation fluid. Introduction of oxygen or nitrogen and eventually addition of glucose then followed. Fifteen min later Compound 48/80 was added, and after a further 20 min, the incubation fluids were withdrawn.

All concentrations of substances mentioned in the text refer to the final concentration in the incubation medium.

Degranulation of mast cells in isolated rat mesentery

The technique used was the same as that described by Högberg and Uvnäs (19 1960). The mesentery pieces were incubated in open bowls on a water bath at 37° C for 20 min after the addition of Compound 48/80 (0.5 μg/ml, unless otherwise indicated). Where inhibitors and glucose were investigated, the mesentery was preincubated together with these substances before the addition of Compound 48/80 as described in the text.

Table I. The effect of oxygen, nitrogen and glucose (5.6 mM) on Compound 48/80 (35 μg/ml) induced histamine release from rat lung tissue in vitro. Histamine release computed in per cent of total histamine content. Spontaneous release deducted from all values

Exp. no.	Histamine release (A)	(B)	(C)	(D)
1	34.1	27.7	28.8	1.0
2	27.1	23.3	26.1	1.4
3	30.7	28.6		
4	36.8		35.3	1.9
5	26.7	19.4	25.9	1.4
6	32.5	19.3	30.4	2.3
Mean and standard error of mean	31.3 ± 1.6	23.5 ± 2.0	29.3 ± 1.7	1.6 ± 0.23
Significance of difference of mean from O_2 + glucose (t-test)		P = 0.05–0.01	P = > 0.05	P = < 0.001

Incubation procedures
A. 15 min glucose O_2 + 20 min 48/80 O_2
B. 15 min O_2 + 20 min 48/80 O_2
C. 15 min glucose N_2 + 20 min 48/80 N_2
D. 15 min N_2 + 20 min 48/80 N_2

When the influence of nitrogen or oxygen on the degranulation was investigated, the mesentery pieces were incubated in stoppered Erlenmeyer flasks in a Warburg apparatus at 37° C without shaking. The incubation fluids were nitrogenated or oxygenated for at least 10 min, before the addition of the mesentery pieces. After these had been added, the needles used for gassing were withdrawn so that they were just above the surface of the incubation fluids, in order to avoid mechanical damage of the mesentery cells during incubation. In all the experiments, the mesentery pieces were exposed to Compound 48/80 for 20 min. All values given are the means of duplicates. Further details are given in the text.

Materials

Compound 48/80 was prepared by AB Leo, Hälsingborg, Sweden, and supplied by the courtesy of Dr B. Högberg.

The following carbohydrates were used:

D-glucose, anhydrous (analytical reagent grade, from the Mallinckrodt Chemical Works)
D-galactose (not graded, from Hoffmann-La Roche)
D-fructose + H_2O (not graded, from Hoffmann-La Roche)
Maltose + H_2O (not graded, from Hoffmann-La Roche)
Lactose + H_2O (not graded, from Hopkins and Williams Ltd)
Sucrose (analytical reagent grade, from the Mallinckrodt Chemical Works)

All inhibitors were obtained from standard commercial sources, except for allicin, which was prepared from garlic according to Cavallito and Bailey (1944).

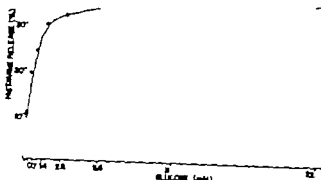


Fig 4 The effect of glucose (concentrations geometrically decreasing from 22.4 to 0.175 mM) on histamine release induced by Compound 48/80 (35 µg/ml) from rat lung tissue under the influence of nitrogen. Histamine release computed in per cent of total histamine content. Spontaneous release deducted from all values.

Results

A. LUNG TISSUE

Dose response relationship

Guided by the dose response curve in Fig 1 the histamine dose chosen for the experiments described under A, was 35 µg/ml Since the histamine content of the lung tissue varied considerably from animal to animal it was not surprising to find wide variations between the animals in the amount of histamine released by one and the same dose of Compound 48/80 (Fig. 2) On the other hand the percentual release was fairly constant amounting to 31 4 ± 4 7 per cent (mean and standard deviation in Fig 2) Consequently in the following, histamine release is given in per cent of the total histamine content.

Influence of oxygen nitrogen and glucose

Nitrogen was found to strongly depress the histamine release from rat lung tissue provided that the incubation fluid was glucose-free. In the two experiments shown in Fig 3 the histamine release caused by Compound 48/80 fell from an average of 31 per cent in an oxygenated medium containing glucose 5 6 mM (1 mg/ml) to 1 per cent in a nitrogenated glucose-free medium. On the other hand, in the presence of glucose oxygen lack did not significantly lower the histamine release In glucose free, but oxygenated medium, the histamine release was slightly reduced as compared with that in the presence of glucose and oxygen. The average values from 6 exp. (Table I) show that, under nitrogen and in the absence of glucose (D) the histamine release was depressed to around 1 per cent, as compared with 31 per cent in oxygenated medium containing glucose (A) Under nitrogen, but in the presence of glucose (5 6 mM) (C) the release was not significantly lowered below that of (A) whereas under oxygen but in absence of glucose (B) the release fell to about

Table II The effect of glucose (5.6 mM) on the histamine release from rat lung tissue under nitrogen in vitro induced by Compound 48/80 (35 µg/ml). Histamine release computed in per cent of total histamine content. Spontaneous release deducted from all values

Exp. no.	A	B	C	D
1	39	8	4	
2	45	3	1	
3	35	8	0	
4	36	12	5	
5	31	7	5	
6	36			26
7	33			28
Mean	37	8	3	27

Incubation procedures
A 15 min glucose N + 20 min 48/80 N_2
B 15 min N + 20 min 48/80 glucose N
C 15 min N_2 + 20 min 48/80 N
D 15 min N + 15 min glucose N + 20 min 48/80 N

24 per cent. This fall was significant (P = 0.05 — 0.01) although small compared with the decline in (D).

The minimal concentration of glucose required to appreciably counteract the depressing effect of nitrogen atmosphere was found to be around 0.25 mM (0.045 mg/ml). Above 5.6 mM (normal blood level) increase of glucose concentration did not appreciably enhance the histamine release (Fig. 4).

To obtain the optimal preservative effect of glucose on the histamine-releasing action of Compound 48/80 in nitrogenated lung tissue, preincubation of the glucose with the tissue was required. As shown in Table II when glucose and Compound 48/80 were added simultaneously to the incubation medium (B) the histamine release averaged only 8 per cent as against 3 per cent in the absence of glucose (C), whereas 37 per cent histamine was released when glucose was added 15 min before Compound 48/80 (A). The minimal preincubation time needed for obtaining the full glucose effect, could not be determined with the technique used but 15 min preincubation sufficed to produce a maximal effect. The quantitative evaluation of the preservative effect of glucose was complicated by the fact that incubation of the lung tissue in nitrogenated, glucose-free medium, caused a progressive decline in the restorative ability of glucose. During 15 min incubation under nitrogen in the absence of glucose, this decline was, however, too low to affect the validity of our results (see (D) in Table II).

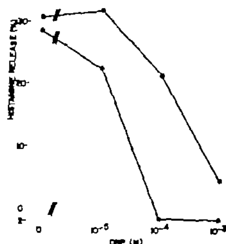


Fig. 5. The effect of DNP on histamine release induced by Compound 48/80 (35 μg/ml) from rat lung tissue under oxygen in presence (•—•—•) and absence (•—•—•) of glucose (5.6 mM). Histamine release computed in per cent of total histamine content. Spontaneous release deducted from all values.

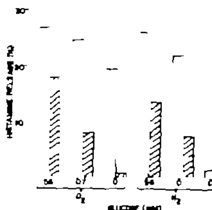
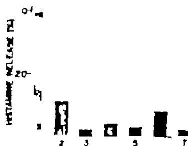


Fig. 6. The effect of DNP (10^{-4}M) on the histamine release induced by Compound 48/80 (35 μg/ml) from rat lung tissue under the influence of oxygen and nitrogen in absence and presence (0.7 and 5.6 mM) of glucose. Unshaded blocks represent histamine release in absence of DNP and shaded blocks that in presence of DNP. Histamine release computed in per cent of total histamine content. Spontaneous release deducted from all values.

The effect of dinitrophenol

Dinitrophenol (DNP) was observed to block the histamine release in concentrations above 10 M provided this reagent was allowed to act on the lung tissue in a glucose-free oxygenated medium. The presence of glucose reduced this inhibitory action of DNP. In the experiment shown in Fig. 5 the histamine release, in the absence of DNP and glucose was 29 per cent. With DNP 10^{-4}M no release occurred; with DNP 10^{-4}M + glucose 5.6 mM 22 per cent histamine was released. Fig. 6 shows that DNP 10^{-4} M reduced histamine release in the presence of glucose (5.6 and 0.7 mM) to the same extent both in an oxygenated and in a nitrogenated medium.

Fig. 7. The effect of mono- and disaccharides on the histamine release induced by Compound 48/80 (35 μg/ml) from rat lung tissue under nitrogen. Histamine release computed per cent of total histamine content. Spontaneous release deducted from all values.
1. Glucose 5.6 mM
2. Galactose 5.6 mM
3. Fructose 5.6 mM
4. Lactose 5.6 mM
5. Sucrose 5.6 mM
6. Maltose 5.6 mM
7. No saccharide present.



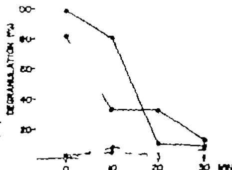


Fig. 8. Degranulating action of Compound 48/80 (—•—•— = 2 μg; —•—•— = 0.5 μg) in nitrogenated glucose-free medium on mesentery mast cells preincubated under nitrogen for various periods. Abscissa represents preincubation time under nitrogen.

The effect of monosaccharides and disaccharides

The restorative effect on the histamine release under nitrogen seems to be more or less specific for glucose. Out of 6 mono- and disaccharides tested, glucose alone had a marked effect. Galactose and maltose, however, were observed to exert a slight effect, whereas fructose, lactose and sucrose were inactive (Fig. 7).

Unfortunately pro analysi products of most of the sugars, including galactose and maltose, were not available in our case. These two sugars were found to be contaminated with glucose. Determinations by the glucose-oxidase method, showed that galactose contained about 5 per cent, and maltose about 7 per cent glucose. The contamination with glucose could account for at least part of the enhancing effects of these sugars. However, considering the high glucose content of the maltose preparation, the enhancing effect of this sugar was surprisingly small. We do not know whether this discrepancy between the glucose content of the maltose and the observed enhancing effect of this disaccharide can be explained as the consequence of substrate competition between glucose and maltose in our histamine-release system, or as a result of an unspecificity of the glucose-oxidase method.

B. MESENTERY MAST CELLS

On the assumption that the effect of oxygen deficiency and the action of glucose on the histamine-releasing property of Compound 48/80 reflected a change in the responsiveness of the mast cells, we extended our studies to

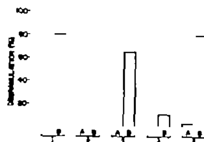


Fig. 9



Fig. 10

Fig. 9 The preservative effect of glucose (5.6 mM) on the mast cell degranulating action of Compound 48/80 (0.5 μg/ml) under nitrogen.
1 20 min 48/80 (without N₂)
2 15 min N + 20 min 48/80 N
3 15 min glucose N + 20 min 48/80 N
4. 30 min N + 20 min 48/80 N
5. 30 min glucose N + 20 min 48/80 N
A Controls in absence of Compound 48/80
B The effect of Compound 48/80

Fig. 10 The effect of glucose (5.6 mM) on Compound 48/80 (0.5 μg/ml) induced mast cell degranulation after various periods of nitrogenation in absence of glucose.
1 15 min glucose N + 20 min 48/80 N
2. 10 min N + 15 min glucose N + 20 min 48/80 N
3. 20 min N + 15 min glucose N + 20 min 48/80 N
4 30 min N + 15 min glucose N + 20 min 48/80 N
5. 60 min N + 15 min glucose N + 20 min 48/80 N
6. 30 min glucose N + 20 min 48/80 N
A Controls in absence of Compound 48/80
B The effect of Compound 48/80

include rat mesentery mast cells. Previous failure (Högberg and Uvnäs 1960) to demonstrate an inhibitory effect of anoxia on the degranulating action of Compound 48/80 on mast cells may have been due to inadequate anoxic conditions or to the presence of intracellular energy-yielding substances enabling the degranulation process to develop anaerobically. New attempts were therefore made with cells preincubated in nitrogenated glucose free medium for various periods. The sensitivity of such cells to Compound 48/80 declined progressively with increasing anoxic preincubation provided that the subsequent exposure to the releaser was performed under continuous anoxia and glucose free conditions. Fig. 8 shows 2 exp in which the mast cells became practically unresponsive to Compound 48/80 within 20—30 min preincubation. There were variations in the length of the anoxic preincubation required to inhibit markedly the mast cell degranulation. In some experiments no preincubation was needed, in others, the inhibitory effect was apparent only after 15—20 min of preincubation.

In the presence of glucose (5.6 mM) the mast cells retained their sensitivity to Compound 48/80 in spite of prolonged exposure to anoxia for at least an hour (15 and 30 min in Fig. 9 and 30 min in Fig. 10).

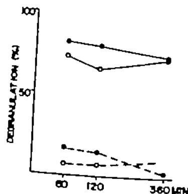


Fig. 11 A

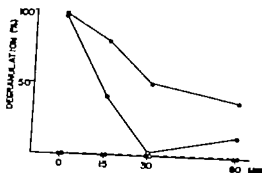


Fig 11 B

Fig. 11 A and B. The effect of preincubation of rat mesentery pieces, under oxygen (A) and nitrogen (B) in absence of glucose, on the degranulation of mast cells induced by Compound 48/80 (0.5 μg/ml) in glucose-containing (5.6 mM) (•—•—•) and glucose-free (•- -•- -•) oxygenated medium. After the preincubation periods the mesentery pieces were incubated further 15 min under oxygen (in presence or absence of glucose) before the addition of Compound 48/80. Broken lines represent degranulation in absence of Compound 48/80. Abscissa represents preincubation time. Note partial restoration in B on further incubation under oxygen in presence of glucose.

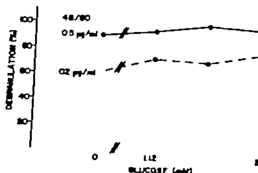


Fig. 12. Mast cell degranulation by Compound 48/80 (0.2 and 0.5 μg/ml) in presence of different concentrations of glucose.

As shown in Fig 10 in order to exert its preservative action, glucose had to be present from the beginning of the exposure to nitrogen. When added to cells preexposed to nitrogen, glucose progressively lost its restoring effect on the degranulating action of Compound 48/80

In contrast to the effect of nitrogen, preexposure of mast cells to oxygen for up to 4 hours did not appreciably reduce the sensitivity of the cells. In oxygen, such cells retained their responsiveness, even in the absence of glucose (Fig 11 A) On the other hand, oxygenation without glucose was unable to restore the inhibitory effect of prolonged nitrogen exposure, while in the presence of glucose a partial recovery occurred, as shown in Fig 11 B.

Glucose did not enhance the degranulating action of Compound 48/80 under aerobic conditions, as shown in Fig 12, where glucose concentrations, varying

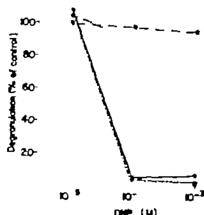


Fig. 15. The effect of glucose (11.2 mM) on the inhibitory action of DNP on degranulation of mast cells by Compound 48/80 (0.5 μg/ml).
—•—•— DNP added 15 min before addition of Compound 48/80 (no glucose present)
—•—•— DNP added 30 min before and glucose 15 min before addition of Compound 48/80
—•—•— DNP and glucose added together 30 min before addition of Compound 48/80
—•—•—• Glucose added 30 min before and DNP 15 min before addition of Compound 48/80
All values computed in per cent of the degranulation found in the absence of DNP

from 0 to 112 mM, *were without appreciable effect on the degranulation*. In this experiment and those subsequently described the original technique devised by Högberg and Uvnäs was employed

When mast cell degranulation was blocked by enzyme inhibitors, glucose was found to counteract the blocking effect caused by some metabolic inhibitors which, it is assumed, interfere with oxidative phosphorylation and respiration (DNP sodium azide, sodium cyanide) For the anti inhibitory effect to develop fully the glucose had to be added before the metabolic inhibitor a fact which is shown in respect of DNP in Fig 15 On the other hand, glucose was unable to counteract the blocking action of thyroxin (Table III) The inhibitory action of ninhydrin allicin, Cu^{++} Pb^{++} and Zn — taken as representatives of inhibitors previously shown to block mast cell degranulation — was not influenced by glucose (Table III)

Discussion

The present observations clearly indicate that histamine release from rat lung tissue, as well as degranulation of rat mesentery mast cells, when induced by Compound 48/80 are energy requiring processes. They are both blocked by anoxia and by metabolic inhibitors. The inhibition is counteracted by glucose indicating that anaerobic pathways are available. The energy-yield from the anaerobic breakdown of glucose seems to offer as favourable conditions for the reactions to proceed as those existing in the presence of oxygen and glucose.

The fact, that histamine release and mast cell degranulation occur under aerobic conditions even when glucose is not added suggests that also other energy donors are operative.

Table III The effect of glucose (11.2 mM) on the action of inhibitors of mast cell degranulation induced by Compound 48/80. "With glucose" denotes addition of glucose (11.2 mM) 30 min and inhibitor 15 min before addition of Compound 48/80 (0.5 μg/ml). "Without glucose" denotes addition of inhibitor 15 min before addition of Compound 48/80 (0.5 μg/ml). All values computed in per cent of degranulation in absence of inhibitor

Inhibitor	Concentration of inhibitor (M)					
	10[illegible]	10[illegible]	10[illegible]	10[illegible]	10[illegible]	10[illegible]
Sodium cyanide						
with glucose			105	106	101	93
without glucose			102	101	49	9
Sodium azide						
with glucose			99	92	75	
without glucose			94	11	9	
2,4-dinitrophenol						
with glucose			100	98	95	
without glucose			108	5	7	
Thyroxine						
with glucose			108	47	9	
without glucose.			97	26	2	
Ninhydrin						
with glucose			101	22	17	
without glucose			94	10	9	
Alloxan						
with glucose	101	52	11	8		
without glucose	91	53	5	3		
Pb						
with glucose	103	89	34	20		
without glucose	106	59	9	8		
Cu						
with glucose	87	65	6	10		
without glucose	104	66	12	3		
Zn						
with glucose		97	70	8	13	
without glucose		104	76	13	8	

Concentration uncertain due to low solubility of thyroxine.

Glucose was able to restore the histamine release inhibited by nitrogenation of the lung tissue, provided that it was added before Compound 48/80 (Table II). Further, in order to counteract the blocking effect of the metabolic inhibitors (DNP, *sodium azide, sodium cyanide*) on the degranulation of mast cells, glucose had to be added before the inhibitors. Since these inhibitory agents attack the metabolic processes at various points, a reasonable explanation, for the required preincubation with glucose, seems to be that this substance is utilized by the mast cells, yielding energy through its breakdown.

The inhibition of histamine release from rat lung tissue by DNP was counteracted by glucose, in spite of preincubation with DNP before the addition of glucose. This is in contrast to the results of the corresponding experiments on mast cell degranulation, where glucose was found to have no preservative effect, if DNP was added before the glucose. The reasons for this discrepancy is obscure but probably it is more of a quantitative than a qualitative nature and is possibly due to th different experimental conditions.

After preincubation under nitrogen in a glucose-free medium the restorative effect of glucose on the mast cell degranulation decreased progressively with the incubation time under nitrogen, provided that the subsequent exposure to Compound 48/80 was performed under continuous anoxic conditions. When the mesentery pieces were correspondingly preincubated under nitrogen in a glucose free medium, a subsequent shift from nitrogen to oxygen did not restore the responsiveness of the mast cells to Compound 48/80. The sensitivity of the mast cells to Compound 48/80 still showed a steep fall with increasing preincubation time under nitrogen. If however glucose (5.6 mM) was added at the same time as oxygen *was substituted for nitrogen* the mast cell degranulating effect of Compound 48/80 was partly restored. This is in accordance with earlier findings (DIAMANT 1960) on the histamine release induced by Ascaris extract from rat lung tissue, where the inhibitory effect of preincubation under nitrogen in a glucose free medium was investigated.

DNP is supposed to block, preferably oxidative phosphorylation. But, as shown in Fig. 6 DNP also blocks histamine release in rat lung tissue under nitrogen, in which histamine release has been restored by glucose indicating that DNP also inhibits glycolytic energy production. The same inhibition occurs under oxygen (Fig. 6) where DNP blocked oxydative phosphorylation is counteracted by addition of glucose. Evidently in these experiments, oxidative phosphorylation *is blocked under aerobic conditions* by DNP and under anaerobic conditions by lack of oxygen. When oxidative phosphorylation is blocked in these ways, DNP is still able to counteract the preservative action of glucose on the histamine release, possibly by inducing ATP ase activity (LARDY and WELLMAN 1953). The inhibitory action of DNP is dependent on the glucose concentration diminishing with increasing glucose concentration.

The inhibitory action of thyroxin on the mast-cell degranulation was not abolished by glucose. It is obscure whether this difference between thyroxin and the other metabolic inhibitors employed is qualitative or only quantitative

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Since this manuscript was accepted for publication A. M Rothschild, I. Vugman and M. Rocha Silva reported in *Biochem. Pharmacol.* 1961 *7* 248—256, that histamine release from rat diaphragm *in vitro* induced by Compound 48/80 was blocked by metabolic inhibitors (DNP and NaCN) and by anoxia. The histamine release was partly restored on addition of glucose.

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From the Histological Department, University of Uppsala, and the Departments of Physiology and Clinical Biochemistry Royal Veterinary School, Stockholm 51 Sweden

Aspects of the Glucose and Amino Acid Metabolism in the Liver and the Diaphragm of Normal and Obese-Hyperglycemic Mice

By

Bo Hellman, Stig Larsson and Sigrid Westman

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Abstract

Hellman, B., S. Larsson and S. Westman. *Aspects of the glucose and amino acid metabolism in the liver and the diaphragm of normal and obese-hyperglycemic mice* Acta physiol. scand. 1961. 53. 330—338. — The utilization of uniformly ^{14}C-labelled glucose was studied in the liver and diaphragm in the American variety of the hereditary obese-hyperglycemic syndrome of mice. Only those obese animals were studied, where the livers were without obvious fatty degeneration. There was higher glucose uptake per liver in the obese-hyper glycemic syndrome. Expressed per unit weight, however no significant differences were found either in the liver or in the diaphragm. The same was true for the formation of CO and the incorporation of glucose in the lipid fraction and the insol ble residue after extraction with TCA and alcohol-ether. An incorporation of measurable amounts of carbon toms from glucose was observed in the following *amino acids in liver alanine nd glutamine, and i the diaphragm,* alanine, glutamine and gl tamic acid. On weight to weight basis there were no differences between normal and obese-hyper glycemic mice, except that in the latter the formation of glutamine was 4—5 times higher. The raised conversion of glucose to glutamine in the liver of obese-hyperglycemic mice was the motiv tion for separate analysis of the activity of glutamic-oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) where however no statistical differences were bserved between the tw types of mice.

Most experimental work ncerning the glucose metabolism in diabetic organisms has been carried ut on animals with experimentally produced diabetes. Alloxan has most frequently been used to induce the diabet c state, because injected parenterally it destroys the B-cells in the islets of Langerhans. Since this type of diabetes is caused purely by an insulin lack, t may be supposed to deviate in many respects from th naturally occurring types of diabetes mellitus. It was therefore of great interest when a strain of mice at the Roscoe B Jackson Memorial Laboratory was found to give rise to offspring with an obese-hyperglycemi syndrome. This syndome is due to the mutation of a single homozygous recessive gene resulting n the expected frequency of 25 % obese offspring (INGALLS, DICKIE and SNELL 1950) In addition to hyper glycemia and obesity these mice are characterized, *inter alia* by increased lipogenesis even during fasting (BATES, ZOMZELY and MAYER 1955) hyper phagia (MAYER *et al.* 1955) and hyperplasia of the islets of Langerhans with greatly increased amounts of depot insulin (WRENSHALL, ANDRUS and MAYER 1955)

The present experiments were performed on obese-hyperglycemic mice and on their lean litter mates, using essentially the same technique as adopted by CHAIN BELOFF CHAIN and POCCHIARI (1956) in their series of experiments on alloxan diabetic rats. The utilization of glucose *in vitro* in the liver and diaphragm was investigated with particular attention to the formation of CO and the incorporation of the glucose in fat and amino acids. The amino acid metabolism n the liver was further studied by determining the activity of two types of transaminases.

Material and Methods

Thirty male obese-hyperglycemic mice (AO-mice) of the American variety (Roscoe B Jackson Memorial Laboratory Bar Harbor Maine) and 30 of their male lean litter mates (AN-mice) were used for the experiment. The mice, which were more than 6 months old, were allowed free access to food. The AO-mice weighed pproximately 50 g, and the AN-mice about 30 g. Only AO-mice which had no macroscopically obvious liver fattening were utilized for the experiment.

Uniformly ^{14}C-labelled glucose was obtained from the Radiochemical Centre, Amersham, England. The radioactive material was diluted to give specific activity of 2 or 20 μC per mg Two different incubation media were used

1 A modified Krebs-Ringer phosphate buffer pH 7 3, with total glucose 0.0056 M (radioactivity = 0.56 mC/mM) Since according to KREBS (1950) high phosphate concentration is incompatible with the physiological concentration of calcium ions, the calcium conten was reduced by one third of its original value

2 A phosphat buffer NaCl 0 098 M, KCl 0 027 M, MgSO 0.0012 M, KH_2PO 0.0004 M, Na_2HPO 0 0175 M and total glucose 0.0056 M (radioactivity = 5.6 mC/mM) pH 7.3 (CHAIN *et al.* 1960)

The animals were killed by ervical dislocation and the tissue samples to be studied were dissected out as quickly as possible. After weighing, the samples were transferred to Warburg vessels, with total volume of bout 5 ml, which contained 0.5 ml of

Table I. µg glucose utilized in different fractions of the liver expressed per 100 mg wet weight. Glucose concentration of the medium 0.1 %, total radioactivity 1 µC (~160 000 cpm). In the Table are also given the body weights in g and the liver weights in mg (wet and dry weight) and the water content of the liver (%). Mean values ± S.E. The figures within brackets denote the number of animals studied

	Body weight	Liver			µg glucose utilized per 100 mg liver (wet wt.) in different fractions			
		wet weight (mg)	water	dry weight (mg)	TCA	Lipid	Residue	CO
AN-mice (12)	31.6 ±1.3	1,586 ±93	68.8 ±0.3	49.5 ±28	68.0 ±2.8	19.2 ±1.9	4.9 ±0.4	5.2 ±0.7
AO-mice (12)	48.9 ±1.4	2,393 ±97	66.0 ±0.6	819 ±46	74.8 ±3.8	21.8 ±1.8	5.0 ±0.8	4.8 ±0.5

medium 1 or 2. The liver slices weighed approximately 70 mg. The centre well of the vessels contained small rolls of filter paper soaked with 30 % NaOH to absorb the respiratory CO_2. Independent of the medium used, the samples were incubated for one hour at 37° in a gas phase of pure oxygen. After the incubation the Na_2CO_3 formed was collected and precipitated with $BaCl_2$, as described by VILLEE and HASTINGS (1949). The radioactivity was measured as $BaCO_3$. In the incubation experiments the remainder of the liver was used for water and fat determinations. The water content was determined by simply drying the tissue at 40° C until a constant weight was reached. The dried tissue was then extracted in a Soxhlet apparatus with petroleum ether to obtain the fat.

Liver slices from 24 animals were incubated in medium 1. After incubation the liver slices were homogenized and three fractions prepared as described by HELLMAN, LARSSON and WESTMAN (1961). The fractions were 1) trichloracetic acid (TCA) soluble substances (e. g. glycogen and low molecular weight substances such as amino acids and intermediates in carbohydrate metabolism), 2) a lipid fraction and 3) insoluble residue (e. g. proteins, nucleoproteins and nucleic acids).

For incubation in medium 2 liver slices and also the diaphragms from 24 animals were used. After one hour of incubation the tissue was treated as earlier described by BELOFF-CHAIN et al. (1955). The samples were homogenized and extracted with 60 % ethanol. The dried extracts were diluted to known volumes and transferred to paper for chromatography as described earlier by CHAIN, LARSSON and POCCHIARI (1960). Chromatography was also applied to the medium. All chromatograms were scanned quantitatively by a modification of the automatic device used by FRANK et al. (1959). The insoluble residues, left after the extraction with the aqueous alcohol, were treated with 0.05 N NaOH at 40° C for 8 hours and then transferred to planchets and measured for radioactivity.

In addition to the incubations in a glucose-containing medium, the glutamic-oxalacetic (GOT) and the glutamic-pyruvic (GPT) transaminase activities in the liver were determined from further 12 animals. The tissues were homogenized in 0.1 M phosphate buffer (pH 7.4) and the enzyme activities were determined by the method described by REITMAN and FRANKEL (1957).

Table II. µg glucose utilized in different fractions of the liver. The figures are calculated on the basis of Table I and give the TCA/100 mg dry wt., lipids/100 mg fat and residue/100 mg fat free dry weight. Mean values ± S.E. The figures within brackets denote the number of animals studied

	TCA/100 mg dry wt.	Lipid/100 mg fat	Residue/100 mg fat free dry wt.
AN-mice (12)	218.1 ±9.2	335.0 ±43.5	19.2 ±1.6
AO-mice (12)	220.1 ±11.0	366.0 ±26.4	18.5 ±2.5

Results

The values for livers incubated in medium 1 are given in Table I. Both the body weights and the liver weights were considerably higher in the AO-mice. The livers of the latter animals had slightly lower but statistically significantly decreased water content ($t = 4.52$, $P < 0.001$). Calculated per unit wet weight, there were no significant differences between AN- and AO-mice with regard to the radioactivity found in different liver fractions. In Table II the values given in Table I are expressed differently. The radioactivity of the TCA-fraction is given as µg glucose utilized per 100 mg liver dry weight, the lipid fraction as µg glucose utilized per 100 mg fat, and the insoluble residue as µg glucose utilized per 100 mg fat free liver dry weight. Expressed in this way there were still no significant differences between livers from AN- and AO-mice.

Table III gives the results of incubating liver slices in medium 2. As regards the conversion of glucose to CO_2, the values were significantly lower than in medium 1 although there were no differences between the AN- and AO-mice. For the lactic acid in the medium there was no significant difference between

Table III. Utilization of glucose in liver slices from AN- and AO- mice. The results are expressed as µg glucose converted per 100 mg of tissue (wet wt.) after 1 hour of incubation at 37° C in O in 0.5 ml medium. Glucose concentration 0.1 %, total radioactivity 10 µC (= 1 600 000 c/min) Mean values ± S.E. The figures within brackets denote the number of animals studied

	CO	Lactic acid	Alanine	Glutamine
AN-mice (12)	2.55 ±0.19	25.7 ±3.2	0.3 ±0.2	0.6 ±0.2
AO-mice (12)	2.84 ±0.36	17.6 ±7.0	0.5 ±0.2	2.5 ±0.6

Table I. μg glucose utilized in different fractions of the liver expressed per 100 mg wet weight. Glucose concentration of the medium 0.1 %, total radioactivity 1 μC (=160,000 c/min). In the Table are also given the body weights in g and the liver weights in mg (wet and dry weight) and the water content of the liver (%). Mean values ± S.E. The figures within brackets denote the number of animals studied

	Body weight	Liver			μg glucose utilized per 100 mg liver (wet wt.) in different fractions			
		wet weight (mg)	water	dry weight (mg)	TCA	Lipid	Residue	CO_2
AN-mice	31.6	1,586	68.8	495	68.0	19.2	4.9	5.2
(12)	±1.3	±93	±0.3	±28	±2.8	±1.9	±0.4	±0.7
AO-mice	48.9	2,393	66.0	819	74.8	21.8	5.0	4.8
(12)	±1.4	±97	±0.6	±46	±3.8	±1.8	±0.8	±0.5

medium 1 or 2. The liver slices weighed approximately 70 mg. The centre well of the vessels contained small rolls of filter paper soaked with 30 % NaOH to absorb the respiratory CO_2. Independent of the medium used, the samples were incubated for one hour at 37° in a gas phase of pure oxygen. After the incubation the Na_2CO_3 formed was collected and precipitated with $BaCl_2$, as described by VILLEE and HASTINGS (1949). The radioactivity was measured as $BaCO_3$. In the incubation experiments the remainder of the liver was used for water and fat determinations. The water content was determined by simply drying the tissue at 40° C until a constant weight was reached. The dried tissue was then extracted in a Soxhlet apparatus with petroleum ether to obtain the fat.

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For incubation in medium 2, liver slices and also the diaphragms from 24 animals were used. After one hour of incubation the tissue was treated as earlier described by BELOFF-CHAIN et al. (1955). The samples were homogenized and extracted with 60 % ethanol. The dried extracts were diluted to known volumes and transferred to paper for chromatography as described earlier by CHAIN, LARSSON and POCCHIARI (1960). Chromatography was also applied to the medium. All chromatograms were scanned quantitatively by a modification of the automatic device used by FRANK et al. (1959). The insoluble residues, left after the extraction with the aqueous alcohol, were treated with 0.05 N NaOH at 40° C for 8 hours and then transferred to planchets and measured for radioactivity.

In addition to the incubations in a glucose-containing medium, the glutamic oxalacetic (GOT) and the glutamic pyruvic (GPT) transaminase activities in the liver were determined from a further 12 animals. The tissues were homogenized in 0.1 M phosphate buffer (pH 7.4) and the enzyme activities were determined by the method described by REITMAN and FRANKEL (1957).

Discussion

It is evident from previous in vitro studies that glucose utilization is reduced in alloxan diabetes. RENOLD et al (1953) and RENOLD HASTINGS and NESBETT (1954) thus showed that the glucose uptake and phosphorylation is lower in liver slices from alloxan diabetic rats. A corresponding decrease of the glucose uptake was also found in the skeletal musculature (VILLEE and HASTINGS 1949 and BEATTY et al. 1959). In view of these observations of a decreased glucose utilization in alloxan diabetes, it is therefore of interest that in the present experiment no significant differences were recorded regarding the glucose utilization per unit weight either for the liver or the diaphragm in the obese hyperglycemic syndrome.

In the case of the liver this was true for the different fractions analyzed, regardless of whether the values were calculated per unit wet or dry weight, or whether the fat content of the liver was taken into consideration. On the other hand it is also worthy of note that there was, in vitro no increase in incorporation in the glycogen-containing TCA fraction, since SHULL and MAYER (1956) reported, after intraperitoneal administration of uniformly ^{14}C-labelled glucose, that the turnover of liver glycogen was approximately 3 times as great per unit liver weight in the AO-mice, in spite of the fact that the glycogen concentration was the same in the two groups. The absence of any differences in the glucose utilization per unit weight of liver and diaphragm in our experiments should further be considered with reference to the condition, that the glucose concentration in the incubation medium was the same for both groups and thus considerably less than the blood sugar values for obese-hyperglycemic mice with free access to food (MAYER, BATES and DICKIE 1951).

While a raised glycogen content of skeletal muscle has been reported in the obese-hyperglycemic syndrome (SHULL and MAYER 1956) there was no previous data about the in vitro glucose utilization of the diaphragm in AO-mice. It is of interest with regard to the absence of a significant decrease in the glucose uptake by the diaphragm of the AO-mice that BELOFF CHAIN et al. (1955) did not either find any changes in rats, which had a less advanced degree of alloxan diabetes. In assessing the results, it should furthermore be recalled that they do not directly reflect the true rates of oxidation since a considerable and quantitatively undetermined dilution of the isotope takes place during the incubation, primarily through glycogenolysis and gluconeogenesis.

The synthesis of lipids constitutes an important part of liver function. The first indication that this was disturbed in alloxan diabetes was found by STETTEN and BOXER (1944) who observed during in vivo experiments an almost complete inhibition of fatty acid synthesis from glucose in the liver of alloxan diabetic rats. These results were confirmed and extended by CHERNICK and CHAIKOFF (1950) CHERNICK et al (1950) by in vitro experiments with C-

labelled compounds. According to these authors, in alloxan diabetes the synthesis of fatty acids from glucose is reduced more than, for example, the oxidation of the glucose to CO_2. The available information concerning lipid metabolism in the obese hyperglycemic syndrome is mainly based on *in vivo* studies of the incorporation of C-2-fragments, the condensation of which is a stage in fatty acid synthesis. BATES, MAYER and NAUSS (1955) showed that when ^{14}C-carboxyl-labelled acetate was fed to the AO-mice, these retained significantly more radioactivity in the liver and carcass fat. On the basis of similar experiments with the same precursor GUGGENHEIM and MAYER (195) suggested that a partial block of the C-2 fragment oxidation was the characteristic biochemical lesion in the obese hyperglycemic syndrome. While PARSON and CRISPELL (1955) did not confirm this, HUGHES and TOLBERT (1958) after intraperitoneal and intravenous injections of ^{14}C-methyl-labelled acetate, found that both the rate and cumulative excretion of $^{14}CO_2$ from the acetate were depressed in the AO-mice.

Of further interest is the report of greater hepatic lipogenesis in the AO-mice, both per unit weight of liver and fat free tissue, after incubating liver slices with ^{14}C-carboxyl-labelled acetate (MAYER *et al.* 1955) It is hardly possible to make any direct comparison between these experiments and our studies of lipogenesis, since MAYER *et al.* did not add glucose to the medium. As shown by MILSTEIN and HAUSBERGER (1956) there is a particularly marked reduction in hepatic lipogenesis upon lowering the glucose concentration in the medium. With regard to our finding that in the AO-mice there were differences for the conversion of glucose to fat only in the case of the whole liver and not per unit weight or in "specific activity" this may perhaps have some connexion with the fact that we used only AO-mice, which had no macroscopically obvious signs of liver fattening The absence of real fatty degeneration in the livers of the AO-mice is supported not only by the fat analysis but also by the water content values, where the differences between the two groups, although statistically significant, were relatively small (*cf* FINLAYSON KROOK and LARSSON 1960)

It is known that protein synthesis is subnormal with insulin deficiency but may be restored to normal by insulin administration Insulin alone, added *in vitro* to liver slices from mildly diabetic rats, does not stimulate amino acid incorporation, whereas glucose alone does, and insulin and glucose together have a still greater effect (KRAHL 1960) The existence of a simultaneous hyperglycemia and hyperinsulinism in the AO-mice made it especially interesting to study their protein metabolism as reflected in the formation of amino acids from glucose. After incubation in uniformly labelled ^{14}C-glucose radioactivity was found, in liver in alanine and glutamine, and in the diaphragm, in alanine, glutamine and glutamic acid, for both the AN- and the AO-mice. It was characteristic of the amino acid pattern in the obese-hyperglycemic syndrome, that the rate of formation of glutamine per unit

liver wet weight was 4—5 times greater. The biological importance of this observation is difficult to evaluate. The presence of the enzyme glutamotransferase and reports that the plasma level of glutamine increases with a positive nitrogen balance have been considered as indicative of an essential role of glutamine in protein metabolism (WHITE *et al.* 1954). Since glutamine is easily hydrolyzed to the metabolically active glutamic acid, comparative studies of the reactions in which glutamic acid take part are of great interest. In the present investigation, therefore, the transamination process was taken up as a separate study in which, however, no definite differences were observed between the AO- and the AN mice with respect to the activity per unit liver weight of glutamic-oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT).

The choice of incubation medium significantly influenced the rate of formation of radioactive CO in the liver slices. The formation of labelled CO was thus significantly lower when medium 2, containing, *inter alia*, more K than medium 1 was used. It may be mentioned that the content of K has been previously shown to have considerable effect on the CO production from uniformly ^{14}C-labelled glucose in the brain tissue (CHAIN, LARSSON and POCCHIARI 1960).

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Acta physiol scand. 1961 53. 339—347

From the Department of Pharmacology The Royal Veterinary College Stockholm, and the Department of Cariology Th Royal Dental School, Stockholm, Sweden

A Comparison of the Distribution of Radioactive Fluorine and Calcium by Use of Double-Isotope Autoradiography

By

Lars-Erik Appelgren Yngve Ericsson and Sven Ullberg

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Abstract

Appelgren L. E., Y Ericsson and S. Ullberg. *A comparison of the distribution of radioactive fluorine and calcium by use of double-isotope autoradiography* Acta physiol. scand. 1961 53. 339—347 — The autoradiographic distribution picture has been compared in whole pregnant mice and young rats at short intervals after the injection of fluorine (NaF^{18}) and calcium ($Ca^{45}Cl_2$) The isotopes were injected intravenously on the same animal and selective registration of F^{18} and Ca^{45} was made utilizing the great difference in decay rates Both isotopes, especially Ca^{45} were rapidly taken up from the blood. F^{18} accumulated more rapidly and selectively in hard tissues while Ca^{45} was taken up in appreciable amounts in some soft tissues. The distribution pattern in bone and in dentine was very similar for F^{18} and Ca^{45} F^{18} was taken up in proportionately low concentration in the calcifying molar enamel. Ca^{45} but not F^{18} was taken up in the cartilage of young animals.

A remarkably high accumulation of Ca^{45} was seen in some groups of striated muscles. These muscle groups may be those which were contracted when the lightly anaesthetized animal was sacrificed by immersion in the freezing mixture The dominating excretory organ for F^{18} was the kidney while high concentrations of Ca^{45} appeared in the mucosa and contents of the small intestine

The role of fluorine in general physiology and caries prevention is essentially dependent on the specific uptake of fluoride ions by the calcified tissues of th body Rather farreaching similarities have been noticed in the patterns of uptake of F and Ca^{45} in hard tissues. It is generally considered that the uptake of F in the skeleton is associated with an incorporation in the hydroxy patite crystals, probably through n exchange with OH-ions.

In the present work we have compared the autoradiographic distribution picture in sections through whole pregnant mice and young rats at short intervals after the injection of NaF^{18} and $Ca^{45}Cl_2$.

In order to eliminate disturbing influence from biological variation the distribution of both isotopes has been studied on the same animal — a procedure which does not seem to have been utilized previously and which we have termed double isotope autoradiography.

The widely diverse decay rates (half life of F^{18} 110 min and of Ca^{45} 155 days) form the basis of selective registration of the two isotopes. With appropriate dosage of F^{18} and Ca^{45} the initially dominating F^{18} radiation can be registered during a few hours without detectable interference from Ca^{45}. After the decay of F^{18} the Ca^{45} radiation is registered on a second film during some weeks exposure.

Methods

Radioactive fluoride solutions were prepared according to methods described earlier (Ericsson and Ullberg 1958, Ericsson, Ullberg and Appelgren 1960). These methods include the production of F^{18} by neutron irradiation of lithium hydroxide, purification of F^{18} by distillation and concentration of the activity by evaporation of the distillate. $Ca^{45}Cl_2$ (spec. act. 1.74 C/g) was obtained from O. R. N. L., Oak Ridge, Tenn., USA.

Three albino mice in advanced pregnancy (two days before expected parturition) and two 15-day-old albino rats were used in the experiments. The radioisotopes were administered by injection in a tail vein. The radiofluoride solution was immediately followed by that containing the radiocalcium. The F^{18} dose was about 2 μC per g body weight and the Ca^{45} dose about 0.05 μC per g body weight.

The time interval between injection and sacrifice was 2 min for one of the pregnant mice and for other animals 30 min. The slightly ether anaesthetized animals were sacrificed by immersion in acetone with solid CO_2 added, the temperature being about —78° C.

The frozen animals were sectioned in a refrigerated room at —10° C. Sagittal 20 μ-sections were cut through the whole animals. Some of the sections were rapidly freeze-dried in a modified Glick-Malmstrom apparatus before the autoradiographic exposure. However, in the majority of cases undried sections were used, chemical fogging being avoided by making the exposure at a lower temperature in boxes half-filled with CO_2-snow.

The autoradiography was largely made according to methods described earlier (Ullberg 1958, Ericsson and Ullberg 1958). The films used were Structurix (Gevaert) and Kodirex (Kodak). The exposure time in the case of registration of F^{18} was 3 hours. After a delay of 2 days in order to allow the F^{18} to decay a new exposure was made which gave Ca^{45} autoradiograms after 3—5 weeks.

A 4-hour control exposure after the decay of F^{18} gave no detectable autoradiographic blackening, which proved that the Ca^{45} did not interfere noticeably during the registration of F^{18}.

Results

Fig. 1—4 show the positives of representative autoradiograms. Owing to the lower radiation energy of Ca^{45} the resolution of the Ca^{45} autoradiograms is better than that of the F^{18} autoradiograms.

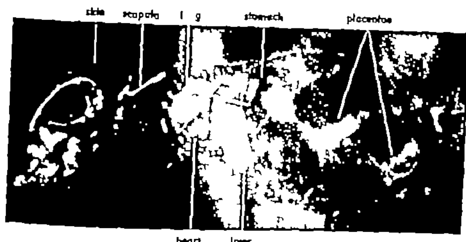


Fig. 1 a. Autoradiogram showing the distribution of F^{18} in a pregnant mouse, 2 minutes after intravenous injection of NaF^{18} + $Ca^{45}Cl_2$. The white areas correspond to high fluorine content. A heavy accumulation in bone can already be seen.

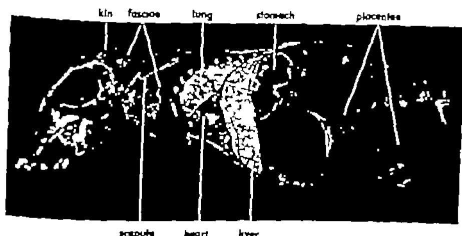


Fig. 1 b. Autoradiogram from the same section as in Fig. 1 a, showing the distribution of Ca^{45}. Note accumulation in bone, lung, muscle fasciae and liver.

Two minutes after the intravenous administration of the two isotopes the blood concentration of both F^{18} and Ca^{45} is still fairly high. (Fig. 1)

F^{18} seems to have been taken up relatively more rapidly in the hard tissues, while Ca^{45} is seen in rather high concentration in some soft tissues. The concentration of radiocalcium in muscle fasciae, lung, skin, and liver is considerably higher than in the blood. None of the isotopes occurs in registrable amounts in the central nervous system and the foetuses show very little uptake.

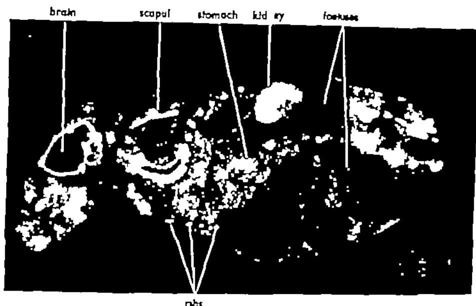


Fig. 2 Autoradiogram of F^{18} in pregnant mouse, 30 minutes after intra enous injection of F^{18} + Ca^{45} The radiofluorine is accumulated selectively in the hard tissues. High concentration is also seen in the renal medulla.

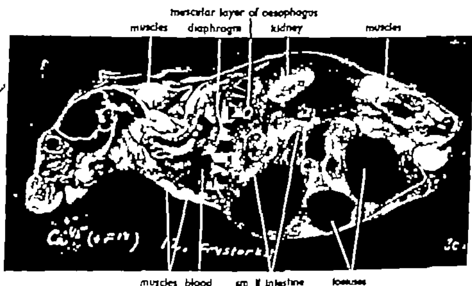


Fig 2 b. Autoradiogram of Ca^{45} from same section as 2 a. The Ca^{45} is cleared from the blood. Accumulation of Ca^{45} is seen in the hard tissues but also in some muscle groups and in the mucosa and contents of the small intestine.

Fig 2 shows the distribution in a pregnant female 30 min after intravenous administration Striking differences now appear in the distribution patterns of F^{18} and Ca^{45}

F^{18} has accumulated selectivel in the hard tissues, which show a ery high concentration. All soft tissues show a low and even concentration of F except the renal medulla where an accumulation can be seen, obviously in connection with excretion of part of the injected radiofluorine (The presence of F^{18} in the gastric lumen, which has never been noticed in our previous F^{18} studies, is probably due to the fact that the mouse has lapped up some urin or urine-conta minated material.) The blood hows bout the same F^{18} concentration as most of the soft tissues.

The Ca^{45} seems to have left the blood almost totally It is, however not at all as selectively located in the hard tissues as is F^{18} An ppreciable concentrat on of Ca^{45} can be noticed in some groups of striated muscles. Thus a very high concentration of Ca^{45} can be seen in the muscular part of the diaphragm, while the tendinous part does not show noticeable quantities of Ca^{45} A high concentrat on of Ca^{45} has been found n the myocardium on other sections.

High concentration of Ca^{45} is seen in some muscle groups in the sternal, neck, and pelvic regions and in some facial muscles like the muscles of the eye and the tactile hairs. In some autoradiograms it is evident that muscle bundles running in one direction show high Ca uptake whil other muscle bundles, crossing the first-mentioned do not show noticeable concentration of Ca^{45} The muscular layer of the oesophagus also shows high Ca^{45} concentration while the concentration in the intestinal muscles is low

The excretion picture is also very different. Whil the F^{18} is excreted mainly in the kidney the Ca^{45} concentration is relatively low in the kidney but high in the intestine, especially in some loops of the small ntestine. The liver shows a lobular pattern in the Ca^{45} autoradiograms and signs of a slight excretion of Ca^{45} via the biliary ducts are seen.

The central nervous system after 30 minutes still shows very low concentration of both F^{18} and Ca^{45} Signs of accumulation of Ca^{45} are, however seen in the choroid plexus. The foetuses show a selective uptake of both F^{18} and Ca^{45} in the mineralised hard tissue portions.

Another pregnant mouse, killed 30 minutes after the injection showed a ery similar distribution of both F^{18} nd Ca^{45} Thus high concentration of Ca^{45} was found in the diaphragm, myocard um and in bout the same groups of skeletal muscles as in the aforementioned case.

In Fig 3 th distribution of F^{18} and Ca^{45} is compared n a 15-day old rat, killed 30 min after intravenous injection.

The F^{18} has as usual accumulated selectively in the hard tissues and is also seen in the kidney

A clear difference can be noted in the distribution pattern in the teeth (see enlargement Fig 4) The F^{18} uptake in the molar enamel is very slight in comparison with the dentine, whil the relative enamel uptake of Ca^{45} is considerably higher This does not, however come out with the unerupted upper incisor

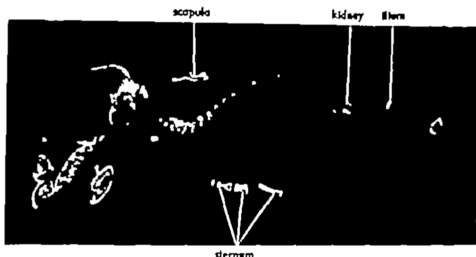


Fig. 3 a. Autoradiogram of F^{18} in a 15 day old rat, 30 minutes after intravenous injection of F^{18} + Ca^{45}. Accumulation of F^{18} can be seen only in the hard tissues and (due to the excretion) in the kidney

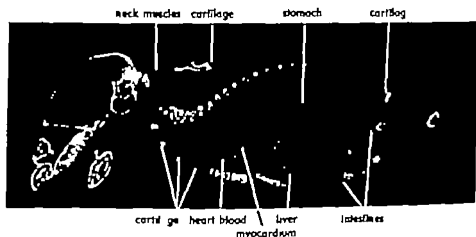


Fig. 3 b. Autoradiogram of Ca^{45} from the same section as 3 a. No Ca can be seen in the blood. The highest uptake can be noted in the hard tissues, but fairly high concentration can also be seen in cartilage some muscles, liver and intestinal mucosa and contents.

In the sternal and rib cartilage an uptake of Ca^{45} is seen, which is lower than that of bone but still marked In the F^{18} autoradiogram no corresponding uptake can be noticed. A similar difference can be seen in the not yet ossified cartilage of the pelvic bone and scapula. A fairly high concentration is also seen in some muscles in the neck region and in the myocardium, but the diaphragm in this case shows a low level

A high concentration of Ca is seen in the li er The intestine, especially the small intestine, shows an accumulation of Ca in ts mucosa and contents.



Fig. 4 a. Autoradiogram of F^{18} from th head of a 15 days old rat, 30 minutes after intravenous injection of F^{18} + Ca^{45}. Note low uptake in the enamel of the upper molars

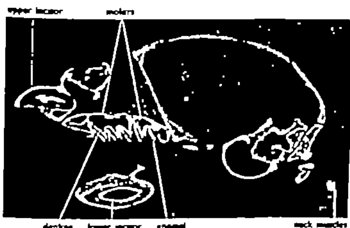


Fig. 4 b. Autoradiogram of Ca^{45} from the same section as 4 a. Note high uptake in the enamel of the upper molars

Discussion

In the above description of results the basis of comparison between F^{18} and Ca^{45} distributions has been the relati e filmblackening by different tissues. The radiation absorption within the sections has been negligible for both F^{18} and Ca^{45} and therefore has not caused appreciable alterations of the relati intensity of radiation among different tissues.

Concerning the distribution in hard tissues the observation of a relatively low uptake of F[18] in the molar enamel agrees with the low concentration of fluorine found in chemical analysis of the enamel as compared with the dentine (review BREDEMANN 1956)

The inference of the observation that Ca^{45} but not F^{18} accumulates in cartilage in young animals may perhaps be that the calcium is not yet incorporated in apatite crystals, which may be a pre requisite for the uptake of fluorine. This may be of importance for estimating the role of high doses of fluorine in sclerotization of cartilage and ligaments and deserves further study

In bone, however F^{18} and Ca^{45} show a more coinciding distribution pattern. It is evident that differences in distribution concern especially the soft tissues.

The most astonishing finding is the high concentration of Ca^{45} in certain muscle groups, especially in view of the low total calcium content found in muscle tissue (EVERETT 1946 GROSSE BROCKHOFF 1950, BRONNER 1958) As the lightly anaesthetized animals made warding-off movements at immersion in the freezing mixture it is conceivable that the muscles which were contracted in the freezing moment may be the ones that show high Ca^{45} content in the autoradiograms.

The finding of Ca^{45} negative muscle bundles crossing Ca^{45}-rich bundles may support this theory The observation may perhaps be put in relation to the finding made in investigations with isolated muscles showing that the strength of a muscle contraction depends on the calcium ion concentration and that a very low calcium concentration completely prevents contraction (PAUSCHINGER and BRECHT 1961)

The possibility that the distribution of Ca^{45} 30 min after intravenous injection is not representative for the distribution of total calcium must be considered. In this connection a recent finding of LIKINS and CRAVEN (1960) may be mentioned Starved rats excreted much less of injected Ca^{45} through the kidneys than normally fed rats in spite of an equally great urinary excretion of chemically analyzed calcium among the two groups of animals. These authors suggested an uneven partitioning of the injected label among the different calcium pools.

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Acta physiol. scand. 1961. 53. 348—365

From the Institute of Biological Chemistry University of Copenhagen, Denmark

Osmotic Behaviour of the Epithelial Cells of Frog Skin

By

Enid A. C. MacRobbie and H. H. Ussing

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Abstract

MacRobbie, E. A. C. and H. H. Ussing. *Osmotic behaviour of the epithelial cells of frog skin.* Acta physiol. scand. 1961. 53. 348—365. — The osmotic behaviour of the frog skin epithelium has been investigated by microscopic measurements of volume under different experimental conditions. Simultaneous measurements of potential and short-circuit current were made. The effects of changes in tonicity of both bathing solutions and of ionic replacements (K^+ for Na^+, SO_4^{--} for Cl^-) were studied. Furthermore the active sodium transport was stimulated by short-circuiting and by application of antidiuretic hormone and inhibited by low pH and by g-strophanthin.

The following conclusions could be drawn.

1) The outward facing boundary of the epithelium is permeable to Na^+ and Cl^- but impermeable to K^+ and SO_4^{--}.

2) The inward facing boundary is permeable to K^+ and Cl^- but practically impermeable to Na^+ and SO_4^{--}.

3) The outward facing boundary is much less permeable to water than is the inward facing one.

4) Application of antidiuretic hormone to the inside bathing solution increases the water permeability of the outward facing boundary whereas the inward facing membrane is unaffected.

5) Inhibition of the active sodium transport by either g-strophanthin or by low pH in the inside bathing solution was accompanied by a pronounced decrease in the passive ion permeabilities.

6) The results strongly indicate that potassium is transported actively from the inside bathing solution into the epithelium.

Present address: Botany School, Cambridge, England.

Table I. Ion concentrations in the Ringer solutions used (mM)

	Chloride Ringer	Sulphate Ringer
Na^+	113.5	113.5
K^+	1.88	5.0
Ca^{++}	1.08	1.08
Cl^-	115	0
HCO_3^-	2.4	2.4
SO_4^{--}	0	58.9

Previous studies have indicated (KOEFOED-JOHNSEN and USSING 1958) that the electric potential across the frog skin can be satisfactorily explained on the basis of the following assumptions:

1) The outward facing boundary of the epithelium is permeable to Cl^- and a few other small anions (Br^-, HCO_3^-) but virtually impermeable to larger anions. It is selectively but passively permeable to Na^+ and Li^+ whereas K^+ and all other cations permeate extremely slowly if at all.

2) The inward facing membrane is permeable to K^+ and Cl^- (and a few other small ions like Rb^+, NH_4^+, HCO_3^-, Br^- etc.) but impermeable to large ions including Na^+, Li^+ and SO_4^{--}.

3) The inward facing membrane is provided with a "sodium pump" which extrudes sodium from the cells, probably in exchange for K^+.

Whereas the existence of the active sodium transport — the "sodium pump" — has been well established (for references see USSING and ZERAHN 1951) it was felt that the specific permeability properties postulated above ought to be verified by observations which are independent of the potential measurements. It seemed likely that a study of the volume changes of the epithelium in response to changes in the composition of the two bathing solutions could provide the desired information about the ionic selectivities of the two epithelial boundaries. Thus, if the inward facing membrane is permeable to KCl but impermeable to NaCl, replacement of the Na^+ of the inside solution by K^+ should lead to diffusion of KCl into the cells and thus to osmotic swelling. The same procedure applied to the outside solution should lead to a slight shrinkage if it is true that the outer membrane is permeable to Na^+ but not to K^+.

Therefore the method described below was developed, permitting accurate microscopic measurement of the volume of the frog skin epithelium under different experimental conditions including changes in tonicity of the bathing solutions as well as ionic replacements (K^+ for Na^+, SO_4^{--} for Cl^- etc.). In addition treatments known to influence the active sodium transport were studied for their effect on cell volume. Thus the sodium transport was enhanced by short-circuiting (USSING and ZERAHN 1951) and by treatment with antidiuretic

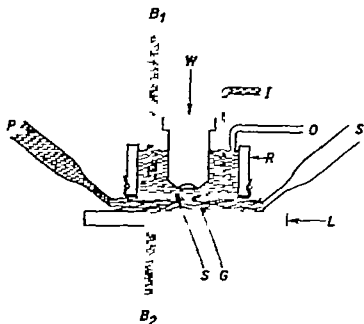


Fig. 1. Diagram of the apparatus for measuring changes in the thickness of the frog skin epithelium.
B_1 and B_2 3 M KCl-agar bridges.
S frog skin surface, inside.
G glass wool pad.
L lucite plate.
R plastic ring.
W water immersion lens of the microscope.
P inflow pipette for "inside bathing fluid".
S outflow pipette connected to suction flask.
I inflow of "outside bathing fluid".
O outflow of "outside bathing fluid".

hormone (FUHRMAN and USSING 1951) and inhibited by low pH (USSING 1949; SCHOFFENIELS 1956) and by treatment with g-strophanthin (KOEFOED-JOHNSEN 1957).

The experimental evidence concerning the ion permeabilities obtained in these studies closely parallels that found by electrical measurements.

Methods

The experiments were done mainly on the abdominal skin of the brown frog, *Rana temporaria*. The frogs were pithed and the skin was then removed and washed in the appropriate Ringer's solution (chloride or sulphate Ringer). The composition of the Ringer's solutions used is given in Table I.

The skin was mounted on the microscope stage as shown in Fig. 1, tied by a cotton thread on a grooved plastic ring (diameter 3 cm, height 1.5 cm) with the outside of the skin facing upwards. The ring was then placed with the inside of the skin in con-

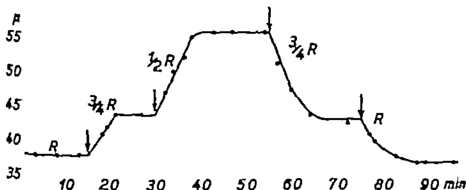


Fig. 2. Volume changes associated with tonicity changes of the inside solution sulphate Ringer. The outside medium is distilled water throughout the experiment.
The total measured thickness of the epithelium in microns is plotted against time in minutes, for various dilutions of sulphate Ringer (R, 3/4 R, 1/2 R)

tact with thin glass wool pad on a shallow lucite dish. A regular flow of solution through the glass wool pad bathed the inside of the skin and a flow was also maintained through the chamber formed by the outside of the skin and the wall of the plastic ring; this allowed rapid changes of the solution bathing both sides of the skin. Volume measurements were made microscopically. The skin was observed through a Leitz water immersion lens in the outside bathing solution (objective magnification × 50 eyepiece magnification × 12.5). The objective was electrically insulated from the body of the microscope by plastic ring. The vertical distance between two reference points, one on the outside surface of the skin and the other on pigment cell immediately below the epithelial cell layer was measured using the calibrated finefocussing control of the microscope. By carefully choosing a spot where sharp line on the surface coincided with a sharp outline of melanophore cell underneath, it was possible to determine this vertical distance between the two levels with an accuracy of bout 1 μ.

Under the conditions of the experiments the area of the skin (and thus of the epithelium) remains constant. Therefore any change in the olume of the epithelium becomes proportional to the change in height. Lateral movements within the observed area are usually nil or quit insignificant, even during gross changes in the cellular volume.

The potential across the skin was recorded continuously during experiments by me ns of an utomatic balancing pparatus constructed and described by Mullen (19) this was calibrated by measuring the potential with high impedanc null-readi g millivoltmeter (PHM 3, Radiometer Copenhagen). The potential measuring electrod in the inside solution consisted of 3 M KCl-agar bridge set in the lucit dis con- necting the glass wool pad with a Ringer-filled vessel cemented to the under-side of the lucite dish, and connected in turn by 3 M KCl-agar bridge to a calomel half-cell. A fine tipped 3 M KCl-agar bridge set in the outside bathing solution was onnected to second calomel half-cell and served as the other potential electrode. I this arrangement relatively low electrode resistances, for use with the recorder (input impedance 30 kΩ) were obtained without excess diffusion of KCl from the agar bridges into he bathing solutions.

Table II

	V (μ)	V (μ)	V_i (μ)	Water permeability of inner membrane (10^{-4} cm/sec.)
Range	35 – 86	12 – 31	16 – 64	15 – 34
Mean (with S. E. and no. of skins in brackets)	58 ± 3 (25)	21 ± 1 (25)	37 ± 2 (25)	24 ± 1 (20)

It was possible to short-circuit the skin in this set-up. The recorder was then used as described by MULLINS, both to maintain the skin with zero potential across it and to record the current necessary to do this. The current electrodes (not shown in Fig. 1) consisted of two platinum wires, one dipping into the outside solution and the other set under the glass wool in a tortuous groove in the lucite plate, arranged to give as uniform coverage of the skin as possible. The variation of the shortcircuit current with the position of the outside potential electrode was, at worst, 5 per cent so that the field over the skin seemed to be reasonably uniform, in spite of the acentric positions in the outside solution of both the outside current electrode and a large conducting body at the microscope objective at its working distance of 0.44 mm. Also the conductivity of Ringer's solution is high enough for the skin to be short-circuited even directly under the non-conducting lens (2 mm diameter) of the objective.

After mounting the skin volume and potential measurements were made for 1–2 hours until the skin had reached a steady state. Only when this level had been firmly established were the experimental conditions changed and the consequent volume and potential changes followed.

Results

Changes in the tonicity of the inside bathing solution sulphate Ringer

With sulphate Ringer inside and sulphate Ringer or distilled water outside, changes in the tonicity of the sulphate Ringer bathing the inside of the skin produced regular and reproducible volume changes in the epithelial cell layer. A typical curve of volume against time in such an experiment is shown in Fig 2. On dilution of the inner solution the cells swelled to a new steady level, and shrank again on return to a more concentrated solution. Judging from the reversibility of the volume changes no solute enters or leaves the cells in the sulphate medium. A slight shift downwards of the steady state level may be seen during the first swelling and shrinking cycle induced in a skin, as if some material were irreversibly lost, but thereafter only water seems to pass the membrane, no matter how often the process of changing the osmotic pressure is repeated. Using the thickness of the epithelium as a measure of the epithelial volume the observations demonstrated that the fractional changes in the latter were considerably smaller than the fractional changes in the tonicity indicating that only some part of the total epithelial volume participated in the volume

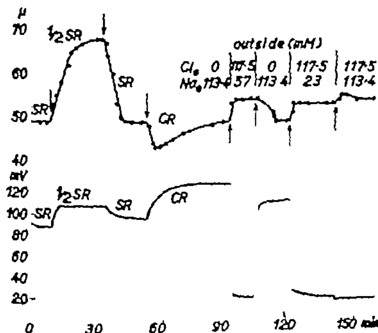


Fig. 1. Volume changes and changes in skin potential associated with changes in the bathing solutions.
Ordinate, upper curve: thickness of epithelium in micra, lower curve: skin potential in millivolts (inside positive).
Abscissa: time in minutes. In the first 95 minutes the outside medium is continuously sulphate Ringer whereas the composition of the inside medium is varied.
First sulphate Ringer (SR) and then in succession 1/2 sulphate Ringer (1/2 SR), sulphate Ringer and chloride Ringer (CR). After 95 minutes the inside medium is chloride Ringer throughout, whereas the outside medium is varied: first modified chloride Ringer with half the Na replaced by potassium, then sulphate Ringer, chloride Ringer with 4/5 of the Na replaced by potassium and finally ordinary chloride Ringer.

changes, whereas the remainder was osmometrically inert. Calling the thickness equivalent of the non-osmotic volume V_n, the thickness equivalent of the osmotic volume V_o and the measured thickness V, the experimental data were found to fit the equation

$$V - V_n = \text{constant tonicity}$$

from which the thickness equivalents of non-osmotic and osmotic volumes could be calculated. This relation was shown to hold in solutions of tonicities relative to sulphate Ringer of 1, 1.35, 2, 0.50, 0.67 and 0.75. The rates of swelling and shrinking in any of these solutions appeared to be governed by a constant permeability factor independent of cell volume. The water permeability of the inner membrane was calculated as the water flux (moles cm^{-2} sec^{-1}) per unit water

Table III

Inside solution	Outside chloride (mM)	Osmotic volume (μ) ~ ?		Cell chloride (mM)	
		Range	Mean	Range	Mean
Ringer	117.5	21 – 38	27 ± 2 (6)	44 – 55	49 ± 2 (6)
Ringer	0	15 – 26	21 ± 1.5 (10)	20 – 41	31 ± 2 (10)

concentration difference (moles cm^{-3}). The above relation between cell volume and tonicity justifies two assumptions on which the interpretation of later results is based, (1) that changes in volume give rise to changes in height of the cells only without any lateral expansion, and (2) that the volume changes are not restricted by elasticity of the cell. Table II summarizes the results obtained in these experiments. V is the thickness equivalent of the osmotic volume in skins pre-equilibrated in sulphate Ringer.

Changes in the tonicity of the outside bathing solution: sulphate Ringer

The volume of the epithelial cell layer in sulphate Ringer solutions appeared to be independent of the tonicity of the outside bathing solution, the volume remained constant whether this was normal sulphate Ringer or distilled water. Since a change of 1 μ in the steady level was easily detectable, the tonicity of the cell bathed in sulphate Ringer inside and distilled water outside was at least 21/22 of that of sulphate Ringer (using the mean value of 21 μ for V, Table II): this puts an upper limit on the water permeability of the skin towards the outside solution, of about 1/21 of that of the inner membrane, or of about $1 \times 10^{-}$ cm/sec.

Volume changes following the substitution of chloride Ringer for sulphate Ringer

When the sulphate Ringer bathing the inside of the skin was replaced by chloride Ringer there was an initial shrinkage, of the rate and amount expected from the relative tonicities of the two solutions (cf. Table I), but this was followed by swelling as chloride entered the cell (see Fig. 3). The final cell chloride concentration could be calculated from the cell volumes and the tonicities of the respective solutions using the equation

$$Cl = (V_c \pi_c - V_s \pi_s)/V_c \quad (2)$$

where V_c and V_s are the stabilized cell volumes in chloride and sulphate Ringer respectively and π_c and π_s are the osmolarities of the corresponding solutions. The equation is derived on the assumption that 1) the cells are Cl-free after the pre-equilibration and 2) that Cl^- enters the cells with an equivalent amount of K^+. It was found that, in general, the cell volume and chloride concentration were independent of the tonicity of the outside solution, but were

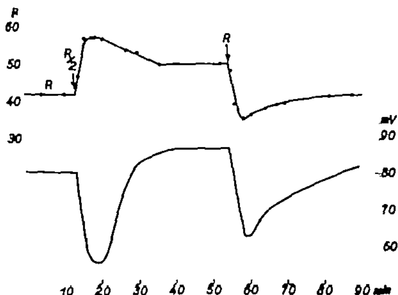


Fig. 4. Volume and potential changes on dilution of the inside medium chloride Ringer. Outside medium 1/20 chloride Ringer throughout.
Ordinate upper curve thickness of the epithelium in micra lower curve skin potential in millivolts.
Abscissa time in minutes. Inside medium first ordinary Ringer (R) then half Ringer (R/2) and finally Ringer again.

dependent of the outside sodium chloride concentration. This is shown in Table III in which the mean volumes and chloride concentrations are given for the skins with chloride Ringer inside, and either high NaCl or no NaCl outside.

Such observations clearly support the contention that the outward facing membrane is permeable to Na^+ and Cl^- but not to SO_4^{--} Fig 3 shows the potential and volume changes associated with changes in the ionic composition of the outside and inside media. In the first 95 min of the experiment the outside medium (sulphate Ringer) is unchanged whereas the inside medium is first sulphate Ringer and then in succession half sulphate Ringer sulphate Ringer and chloride Ringer The slight increase in potential following the dilution of the sulphate Ringer probably is due to the increased potassium gradient cell/inside solution. The potential increase following the shift to chloride Ringer inside reflects the outward diffusion of chloride ions.

After 95 min the sulphate Ringer of the outside solution is replaced by a modified chloride Ringer in which half the Na^+ has been replaced by potas-

Table IV. Swelling after substitution of ½-chloride Ringer for Ringer on the inside

	Initial swelling (μ)	Time to peak (min.)	Subsequent shrinkage (μ)	Time for shrinkage (min.)	Shrinkage as per cent of initial swelling
Range	10 – 20	5 – 13	3 – 13	3 – 17	19 – 70
Mean of 11	15 ± 1	6	7 ± 0.5	14	47 ± 4

Shrinkage on return to chloride Ringer

	Initial shrinkage (μ)	Time to trough (min.)	Subsequent swelling (μ)	Time for swelling (min.)	Swelling as per cent of initial shrinkage
Range	7 – 19	4 – 10	3 – 8	15 – 40	19 – 50
Mean of 10	14 ± 1	7	5 ± 0.5	20	36 ± 3

num. The potential drops precipitously whereas the cells swell, both phenomena indicating that the membrane is readily permeable to chloride. The subsequent ionic replacements in the outside medium show that the phenomenon is reversible and depends on the simultaneous presence of Cl^- and Na. In some skins in very good conditions this volume change with outside chloride concentration was not found. It appeared that a few skins are capable of maintaining a low cellular chloride concentration even with full chloride Ringer outside, either by a lower sodium chloride permeability at the outer membrane, or by a more efficient cation pump. It may be that this capability is more wide spread in skins which have not been exposed to sulphate Ringer for some hours.

Changes in the tonicity of the inside bathing solution. chloride Ringer

In this case the new steady state set up after a change in tonicity of the inner bathing solution is reached by movement of both water and ions, into and out of the cell respectively and therefore the volume changes may be expected to show differences from those in sulphate Ringer where, in the absence of a penetrating anion, only water moves. In the experiments discussed here the outside medium was 1/20 chloride Ringer throughout. A typical curve is shown in Fig. 4 and Table IV collects the characteristics of the curves obtained from 11 frogs.

From these results it is seen that not only is the maximum amount of swelling on dilution of the inside Ringer to half strength considerably less than the

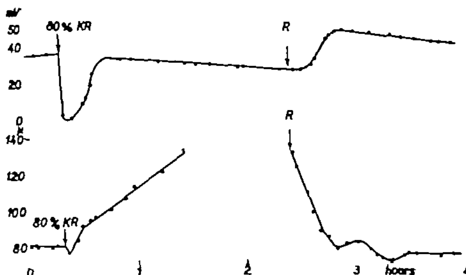


Fig. 5. Volume and potential changes produced by altering the K/Na ratio of the inside chloride Ringer solution (outside medium 1/20 Ringer).
Ordinate, upper curve: skin potential in millivolts, lower curve: thickness of the epithelium in micra.
Abscissa: time in hours.
At zero time the inside medium is ordinary Ringer. At the first arrow this medium is changed to one in which 80 per cent of the sodium had been replaced by potassium (80 per cent KR). At the second arrow the inside medium was changed back to ordinary Ringer.

normal osmotic volume in Ringer (21 ± 2 μ, Table III) but also that the curves show a biphasic course resulting in a pronounced peak, the new steady level being reached after a mean shrinkage of 7 μ. The volume of the cells on return to Ringer shows very similar biphasic changes in reverse. This suggests that the ions whose loss from the cell placed in 1/2 Ringer leads to a reversal of the direction of water movement may be restored to the cell from Ringers. Since it has been shown that the inside surface of the skin is much more permeable to potassium than to sodium (KOEFOED-JOHNSEN and USSING 1958) it is reasonable to assume that KCl is the salt involved in the process. This was confirmed by measuring the K concentration by flame photometry of the solution. Potassium was, indeed, lost from the skin in 1/2 Ringer to the inside solution. The skin was tied on the plastic ring with the inside facing upwards and left to equilibrate for one hour with Ringer inside and 1/20 Ringer bathing the outside of the skin. After a brief rinse on the inside of the skin with Ringer 3 ml of 1/2 Ringer were poured on and left for 1/2 hour

Table V Swelling after substitution of 80 per cent K Ringer on the inside

	Swelling (μ)		Rate of swelling (μ/h)	
	Range	Mean	Range	Mean
Rana temporaria	23 — 48	32 ± 4 (5)	36 — 72	54 ± 6 (5)
Rana esculenta	52, 53	(2)	33 40	(2)

This solution was then withdrawn and analysed for potassium. A loss of potassium of 0 09—0 15 μmoles per cm of skin (4 values) was calculated from the results the loss predicted from the osmotic volumes in Ringer and in 1/2 Ringer is 0 08 ± 0 02 μmoles per cm

The significance of the biphasic course and the peak will be more fully discussed later It was seen in all normal dilution experiments with 1/2 Ringer but could be abolished by poisoning the skin with strophanthin.

The water permeability of the inner membrane in chloride Ringer was estimated from the initial slope of the swelling or shrinkage. The mean value from 12 skins was (23 ± 2) × 10^{-4} cm/sec. Thus there seems to be no significant difference between the water permeabilities of the inner membrane bathed in chloride and in sulphate Ringer solutions. (See, however the discussion.)

Effect of replacing Na by K^+ in the inside solution

When the chloride Ringer bathing the inside of the skin was replaced by a mixture of normal chloride Ringer and potassium Ringer large, regular reversible swelling resulted if the outside solution was distilled water or dilute Ringer solution. The curves obtained in one such experiment are shown in Fig 5 and the results summarized in Table V

With Ringer outside, the effect of substitution of some potassium for sodium in the inside solution was very variable some skins showed little swelling under these conditions, some gave very irregular oscillatory swelling, and a few skins gave smooth curves, but with slower and smaller swelling than with very dilute solutions outside. This can be qualitatively explained on the basis of our assumptions, although the system has too many parameters to permit a quantitative explanation of these experiments as yet.

Volume changes associated with short-circuiting the skin

Some experiments were done in which the volume was measured during alternate periods of normal skin potential and short-circuiting A small reproducible volume change was associated with the change in electrical conditions but both swelling and shrinking were found. Most skins swelled by 2—4 μ on short-circuiting (6 skins) but in 2 experiments a shrinkage of 3—4 μ was observed.

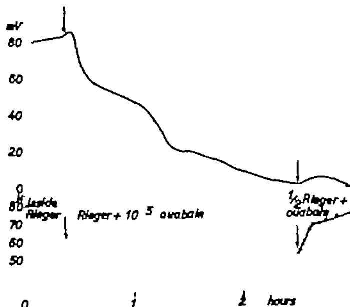


Fig 6. Volume and potential changes produced by ouabain (g-strophanthin) 10^{-5} molar in the inside solution.

Ordinate, upper curve skin potential in millivolts lower curve thickness of epithelium in micra.

Abscissa time in hours.

Outside medium chloride Ringer throughout.

Inside medium initially Ringer

At the first arrow there is switch to Ringer containing 10^{-5} molar ouabain. After the second arrow the medium is 1/2 Ringer with 10^{-5} molar ouabain

Effect of ouabain in the inside solution

Ouabain, in a concentration of 10^{-5} M has been shown to inhibit active sodium transport in the frog skin (KOEFOED-JOHNSEN 1957) and its effects on volume and potential were therefore studied. Typical volume and potential changes are shown in Fig 6 in which ouabain is added to the inside of a skin bathed in chloride Ringer on both sides. The main potential change occurred much earlier than the volume change after a rise of 1—2 mV over the first 3—5 min, there was a rapid decline of potential over the next 7—10 min, followed by a slow fall to zero over several hours. In 6 exp there was no volume change for 30—50 min after dding ouabain, but in 2 exp. there was some swelling during this period of 1 μ and 4 μ respectively In 4 exp. in which no further sol tion change was mad this period of steady volume was followed by shrinkage (of 6 μ 7 μ 8 μ and 13 μ respectively) similar to that shown in Fig 6 Fig 6 lso shows that the characteristic biphasic volume change

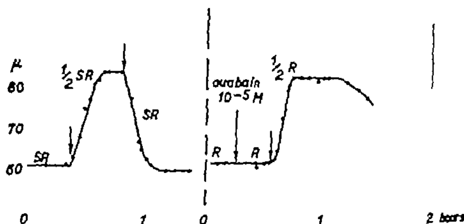


Fig. 7 Swelling of the epithelium in half chloride Ringer (inside) after ouabain treatment, compared with swelling in 1/2 sulphate Ringer
Ordinate thickness of epithelium in micra.
Abscissa time in hours.
I the first experiment shown the outside bathing solution is sulphate Ringer The inside solution is first sulphate Ringer then after the first arrow 1/2 sulphate Ringer and then sulphate Ringer again. In the second run with the same skin the outside and inside media are both ordinary chloride Ringer At the first arrow the inside medium is switched to Ringer containing 10^{-4} molar ouabain. At the second arrow the inside medium is changed to the same concentration of ouabain in 1/2 Ringer

normally produced by application of 1/2 Ringer is replaced by swelling only after treatment with ouabain long enough to produce the above mentioned shrinkage.

During the first period after ouabain treatment the permeability of the inner membrane to KCl is abnormally low as seen from the fact that the cells do not swell as they do in the absence of ouabain with 80 per cent K Ringer inside. The effect on KCl permeability during this period was also shown in 4 exp. in which the swelling in 1/2 Ringer was measured. Fig 7 shows one such curve in which the maximum swelling in 1/2 Ringer is very close to that predicted in the absence of salt movement.

Low pH

Low pH in the inside solution has been shown to inhibit the sodium transport (SCHOFFENIELS 1956) and a concurrent effect on cell volume might therefore be expected No such effect was found. When Ringer buffered to pH 5.9 with 5 per cent phosphate buffer was applied to the inside of the skin the potential fell rapidly but no swelling of the cells was observed. Since inhibition of the pump without swelling suggests that the permeability of the membrane has been greatly reduced, the water permeability was also measured as a functi of pH It was found that at pH 5 9 the permeability of the inner as

Table VI Effect of pH on water permeability

pH	Water permeability of inner membrane (10^{-4} cm/sec.)
5.9	9 ± 1 (7)
8.0	17 ± 1 (10)

to water was reduced to about 50—60 per cent of its value at pH 8, and that the change was reversible. Table VI summarizes the results. (The difference between this value at pH 8 and that quoted earlier is a seasonal difference in the frogs.)

Effects of anti-diuretic hormone

The effects of anti-diuretic hormone were studied by adding insipidin to the inside bathing solution (100 I U per 1) This substance is known to increase the osmotic permeability of the skin while leaving the diffusion permeability to water nearly unchanged, by increasing the pore size in one or more membranes within the skin (KOEFOED-JOHNSEN and USSING 1953)

After treatment of the skin with insipidin it was found that the cell volume in sulphate Ringer was no longer independent of the outside solution. Thus the water permeability of some layer between the epithelial cells and the outside solution must have been increased by hormone treatment. The water permeability of the outside membrane of hormone treated cells was obtained from the initial rate of swelling when distilled water replaced sulphate Ringer on the outside, the inside being sulphate Ringer throughout. The water permeability of the inside membrane was determined from swelling rates as described in p. 9. An independent value for the ratio of the water permeabilities of the two membranes could be obtained from the equilibrium values of the volume changes in these experiments since the degree of swelling must be determined by the condition that the osmotic flow through the outer membrane equals that through the inner membrane. This again requires that

$$P \times \pi_e = P \times (\pi - \pi_e) \text{ or}$$
$$P_o/P = (\pi - \pi_e)\,\pi_e$$

where P is the water permeability of the outer membrane, P that of the inner membrane, whereas π_e is the osmotic pressure in the cell and π_i the osmotic pressure of the inside solution. π is calculated on the assumption that the cellular contents of solutes remains constant even after hormone treatment. The values for the permeability rati calculated according to the latter method were in good agreement with those obtained from the rates of swelling (see Table VII)

Commercial preparation of vasopressin from Alfred Benzon A/S, Copenhagen.

Table VII Water permeabilities of hormone treated skins

Exp.	Measured water permeability inside μ/sec.	Measured water permeability outside μ/sec.	Ratio of permeabilities (from rates of swelling)	Ratio calculated (from degree of swelling)
A	22	6.5	3.4	3.5
B	26	4.1	6.3	7
C	26	2.2	12	13
D	20	2.3	9	4

Discussion

The results of the effects of tonicity changes in sulphate Ringer indicate that of the 58 μ epidermis only 21 μ is osmotically active, the other 37 μ taking no part in osmotic changes. This might be explained if it is assumed that mainly the basal layer of cylindrical epithelium cells, the stratum germinativum, responds to osmotic changes, and that the outer cornified or cornifying cells are osmotically inert either due to low water permeability or to excessive permeability to water and electrolytes or because they are mechanically resistant to volume changes. Furthermore there must be a certain non-osmotic volume in the epithelium due to its contents of protein and other solids. Additional work is needed to clarify the relative importance of these three factors in restricting the effective osmotic volume.

The calculated thickness equivalent of the osmotic volume is in good agreement with the actual thickness of the stratum germinativum as measured in histological specimens (see for instance ENGBÆK and HOSHIKO 1957). The observed value of the water permeability of the inner membrane, 24 × 10^{-4} cm/sec, is about the fifth of that reported by SIDEL and SOLOMON (1957) for the human red cell, but is within the range given by PRESCOTT and ZEUTHEN (1953) for various egg cells. It is also considerably less than the value given by KAMIYA and TAZAWA (1956) for *Nitella*. It is possible, however that the method for measuring the rate of swelling underestimates the water permeability of the inner membrane since the connective tissue of the skin is interposed as a slowly mixing layer of 0.2 to 0.5 mm thickness between the bathing solution and the epithelium. The diffusion delay in this layer for Na has a half time of 0.7 to 1.0 min (HOSHIKO and USSING 1957) which is not far from the half time for osmotic swelling of the epithelial cells.

The results of changes of the external solution show that it is the NaCl concentration of the outside solution rather than its tonicity which influences the cell volume. It could, however be the intercellular cement round the outer cornified cells rather than the outer epithelial cell membranes proper which is responsible for the (very low) water permeability of the skin to the outside.

The increase in water permeability after hypophysin treatment must be produced by an increase in pore size in some layer towards the outside of the skin, even though the hormone is effective only when applied to the inside of the skin.

The biphasic form of the curves obtained by tonicity changes in the inside chloride Ringer solution has important interpretations. In 1/2 Ringer the shrinkage following the initial water entry implies that at the peak, although water is temporarily in equilibrium across the inner membrane potassium chloride is not, and the cellular product $K_c \times Cl_c$ is higher than the product $K_i \times Cl_i$ in the inside bathing solution. This must mean that initially $K_c \times Cl_c$ was higher than $K_i \times Cl_i$ in Ringer meaning that KCl was not in equilibrium across the inner membrane but was higher in the cell than the equilibrium level. [At the peak the total salt concentration of the cell has been reduced to half its initial value in Ringer but the permeable ions K^+ and Cl^- have been reduced to less than half their initial sum since the osmotic volume has been less than doubled. A mathematical consequence of the relations $(K_c + Cl_c)_{peak} < 1/2\,(K_c + Cl_c)_{initial}$ and $(K_{c\,(initial)} - K_{c\,(peak)}) = (Cl_{c\,(initial)} - Cl_{c\,(peak)})$ is the product relation $(K_c \times Cl_c)_{peak} < 1/4\,(K_c \times Cl_c)_{initial}$. This then gives the inequalities $1/4\,(K\,Cl_c)_{initial} > (K\,Cl_c)_{peak} > (K\,Cl_i)_{1/2\,Ringer} = 1/4\,(K\,Cl_i)_{Ringer}$ or $(K\,Cl_c)_{initial} > (K_i Cl_i)_{initial}$.]

The cellular product $K\,Cl_c$ can only be maintained higher than the external product $K_i\,Cl_i$ by an active transport of either K^+ or Cl^- from the inside solution into the cell, and by a passive replenishment of the other ion. Both Na and Cl^- are lower in the cell than in Ringer and therefore the concentration gradients are right for a passive entry of chloride to the cell as NaCl enters through the outside membrane, and to a much lesser extent through the inside membrane if this is somewhat permeable to sodium. Therefore it is possible to achieve this non-equilibrium state by an active transport of potassium into the cell from the inside solution. The possibility that the high cellular product $K\,Cl$ is maintained by an active transport of chloride from the inside solution into the cell may be discarded as there is no other anion available to produce a passive entry of potassium to the cell, against its very considerable concentration gradient, through either membrane. The volume changes and the potential changes produced by diluting the inside solution showed similar time courses, but one point should be noted from Fig. 4. When water is moved into the cell the potential rises as KCl enters the cell and falls as KCl leaves the cell, but the reverse is true when the flow of water is outward in this case the potential rises as the cellular KCl decreases and falls if the cellular KCl is increasing. This suggests that the relative permeabilities of the inner membrane to K^+ and Cl^- somehow depend on the direction of water flow. An inward flow of water increases the ratio $P_K\,P_{Cl}$ whereas under conditions of outward water flow the membrane permeability to chloride is high relative to its potassium permeability.

The swelling produced by a replacement of some Na by K in the inside

bathing solution is to be expected from the model with the inside membrane permeable to potassium but only very little permeable to sodium.

The different time course of the swelling and the shrinking in Fig 5 can be predicted on theoretical grounds Since the cellular product $K_c \times Cl_c$ is maintained higher than the product $K_i \times Cl_i$ of the inside solution the gradient of KCl producing the volume change is not simply the change in the external product $K_r \times Cl_r$. For the swelling the effective gradient is less than the external change by an amount δ the difference between $K_c \times Cl$ and $K_i \times Cl$ in Ringer and for the shrinkage the effective gradient is greater than the external change by an amount δ the difference between K_c Cl and K_i Cl_i in 80 per cent K Ringer On this account one would expect the shrinkage to be much faster than the swelling as is indeed observed, but the possibility remains that the ionic permeabilities depend on the direction of the gradient.

The effect of short-circuiting must depend on the relative ion permeabilities of the two membranes, and there would seem to be enough variables to explain, qualitatively both swelling and shrinkage as found in the experiments. At the moment a quantitative description of these changes does not seem possible.

Stoppage of the pump, unaccompanied by any permeability changes can only lead to swelling of the cells, since NaCl continues to leak into the cell from the outside and the initially balanced diffusion of KCl to the inside solution can only decrease with time. Therefore the lack of volume changes during the first hour or so of ouabain treatment can only be explained on the basis of permeability changes as well as stoppage of the pump, and suggest that both membranes become relatively tight to ions during this period. The tightness of inner membrane is shown by the experiments with 80 per cent K Ringer and 1/2 Ringer in this period The fact that the ouabain treated skin responds to 80 per cent K Ringer on the inside by a potential drop (like normal skin) shows that at the inner membrane $P_K > P_{Cl}$, and therefore the rate of KCl movement will be limited by P_{Cl}, the lower of the two ion permeabilities. Hence the reduced permeability to KCl implies a reduced chloride permeability of the inner membrane, an effect confirmed by the fact that the behaviour of the inner membrane is closer to that of an ideal potassium electrode after ouabain than before. During the second period in ouabain the KCl permeability of the inner membrane appears to be restored or increased, but to account for the shrinkage it appears that the outer membrane is still tight to NaCl.

Similar permeability changes seem to be associated with inhibition of the pump by low pH in the inside solution, without any concurrent swelling This, together with the results of the water permeability of the inside membrane at low pH suggests that the tightening is a general one to both water and ions. It may be that any interference with the pump mechanism is accompanied by a decrease in the passive permeabilities to ions and that the active ion transport and the passive fluxes are not entirely independent.

Although in most cases a quantitative explanation of the results cannot be produced from so many variables, it does seem from these experiments that measurements of volume changes in the epithelial cells, in conjunction with electrical measurements, does provide useful information on which to base any interpretation of the processes concerned.

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From the Division of Food Chemistry, Royal Institute of Technology and the Departments of Physiology and Clinical Biochemistry, Royal Veterinary School, Stockholm, Sweden

A Comparison of the Efficiencies of Free Lysine and of Roller Dried Skim Milk, Fish Protein and Soya Bean Protein for the Supplementation of Wheat Bread

By

L. E. Ericson, S. Larsson and G. Lid

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Abstract

Ericson, L. E., S. Larsson and G. Lid. *A comparison of the efficiencies of free lysine and of roller-dried skim milk, fish protein and soya bean protein for the supplementation of wheat bread.* Acta physiol. scand. 1961. 53. 366—375. — Free L-lysine HCl, roller-dried skim milk, fish protein and soya bean protein were added to the dough of wheat bread and their supplementary values for the improvement of the protein value of the bread were compared. The growth rates and nitrogen efficiency ratios of young albino rats were used as indices of the improvements obtained.

Within the concentration range studied, the supplementary values of the added substances were directly proportional to the amount of available lysine which they provided. No beneficial effect from the other essential amino acids supplied by the proteins tested as supplements was detected. The data also indicate that roller-dried skim milk suffered a considerably greater loss of lysine during the baking of wheat bread than did the other supplements.

The principle of amino acid supplementation of low quality protein is to add such an amount of the most limiting amino acid that it increases the total content of this acid in the diet to a level where — from a nutritional point of view — it balances the second most limiting amino acid. Due to the

law of diminishing returns" which in this case may be understood and interpreted in terms of the laws of enzyme kinetics, it is uneconomical from a practical point of view to add exactly this amount a slightly smaller quantity will give an optimum result. No beneficial effect should in such a situation result from the addition of the second most limiting amino acid or from other added amino acids. One would therefore, at least as a first approximation, assume t to be unimportant whether or not the most limiting amino acid was supplied free or protein-bound.

In connection with experiments on the amino acid supplementation of wheat bread, we have also studied the supplementary value of a few proteins, viz. milk, fish and soya bean proteins. The results of comparisons of amino acid and protein supplementations will be presented in this paper and discussed in the light of the principles mentioned above. Other factors that might influence the outcome and interpretation of such comparisons will also be discussed.

The growth rates and nitrogen efficiency ratios[1] of young albino rats which wer given the various wheat bread diets were used as indices of the effects due to the supplements. *Both the amino acids, principally lysine, and the* proteins were incorporated into the dough of the bread. This was considered to be a more realistic approach than simply to add the supplements to the diets since in practice, most products to be supplemented would be heat treated in some way *e. g* cooked, baked, fried toasted etc., before being consumed.

Experimental

Analytical methods. The lysine contents of the various dietary ingredients and the *diets were determined microbiologically using Leuconostoc mesenteroides P-60* (ATCC 8042) as the test organism. The Bacto Lysine Assay Medium from the Difco Laboratories, originally developed by STEELE *et al.* (1949) was employed. However the incubation temperature of the tubes was 34 C (SCHIAFFINO McGUIRE and LOY 1958) instead of 37° C as suggested by STEELE *et al* (1949) The content of threonine was also determined in some cases using *Streptococcus faecalis* (ATCC 8043) and the Bacto Threonine Assay Medium (STEELE *et al.* 1949) The details of the procedures used, including the conditions for the extraction and hydrolysis of the samples, are described in a previous paper (ERICSON, LARSSON and LID 1961

Nitrogen was determined by the Kjeldahl method as modified by PERRIN (1953) The ammonia was collected in boric acid and titrated with 0 01 N HCl.

Dry weight determinations were carried out by heating the samples t *104—105* C for 24 h and cooling them in desiccator over silica gel.

Bread formulae and baking conditions. Two different series of rat experiments will be described in the following The basal formula of the bread for the first series was

[1] Nitrogen efficiency ratios (gain of weight per g of nitrogen consumed) were preferred instead of *protein* efficiency ratios partly because the exact correlation between nitrogen and protein for the different ingredients of the diets, such as heat flour milk, fish and soy protein, as not known and partly because the added free amino acids increased the nitrogen but not the protein conten of the diets.

water	1,000 g
wheat flour[1] (70 % extraction)	1,900 g
lard	20 g
roller-dried skim milk (< 1 % fat)	50 g
sugar	10 g
salt	20 g
malt extract	10 g
yeast	200 g

For the second series, which was performed more than a year later the formula was slightly modified partly to suit the quality of the flour then available

water	1,000 g
wheat flour[1] (70 % extraction)	1,850 g
lard	20 g
roller-dried skim milk (< 1 % fat)	50 g
sugar	20 g
salt	20 g
yeast	100 g

These formulae were modified by the addition of amino acids or proteins as will be described later. The amino acids — L-lysine HCl (Pfizer & Co. Inc.) and DL-threonine (Fluka, A. G.) — were dissolved in part of the water for the dough. The proteins — roller-dried skim milk (from the Swedish Milk Products, Stockholm, Sweden) fish protein ("Sunco-protein" from AB Sunco, Mölndal, Sweden) and soya bean protein ("Promine D" from Central Soya Co., Inc., Chicago, USA) — were added to part of the flour before being mixed into the dough. The dry weights, nitrogen and lysine contents for the three proteins tested were determined and found to be 96.8 %, 95.0 % and 92.5 %, 5.60 %, 13.8 % and 13.8 % and 2.86 %, 7.48 % and 5.92 %.

All bread was pan baked. Dividing, rounding and moulding were done by hand. [Proof]ing took place at 45 °C for about 30—50 min and at a relative humidity of ap[proxi]mately 85 %. The baking temperature was 190—220 °C and the baking time [4]0—50 min. Each loaf of bread weighed about 400 g.

After baking, the bread was sliced and dried. In the first series of experiments this took place at 25—27° C for 2 days. The breads used in the second series were dried at 25—40° C for 2 days. The air-dried bread was finally ground in an "Electrolux Assistent" bread mill. The dry weight of the ground bread was approximately 92 %.

The diets. The diets used in both series of experiments were composed of 91 % of the dried and ground bread, 3 % of a salt mixture (HEGSTED *et al.* 1941), 5 % soya bean oil, 0.5 % cod liver oil and a vitamin mixture (HARPER *et al.* 1955). The vitamin mixture provided in mg per 100 g of ration: thiamin HCl 0.5, riboflavin 0.5, niacin 1.0, calcium pantothenate 2.0, pyridoxine 0.25, biotin 0.01, pteroylglutamic acid 0.02, cyanocobalamin 0.002, inositol 10, menadione 0.5 and choline chloride 150. Before being mixed into the rest of the diet, the vitamins were distributed in the salt mixture and a small quantity of finally powdered bread. The cod liver oil supplied approximately 375 I. U. vitamin A and 37.5 I. U. vitamin D per 100 g of diet.

Conditions of the rat experiment. In the first series of experiments, the animals were housed individually in cylindrical glass vessels having a diameter of 20 cm and containing wood shavings. The temperature in the animal room was 27° C. The rats were males of the Wistar strain with an average initial weight of 76 g.

[1] Alexandra, Kungsörnen AB, Stockholm, Sweden.
Daughter Company of AB Astra, Södertälje, Sweden.

Table I Composition of diets and growth rates for the groups of rats in Series I Twelve rats per group were used initially The additions of roller-dried skim milk, L-lysine HCl and DL-threonine were made before baking

Group	Diet Additions expressed in per cent of the fresh weight of the flour	Determined content of Nitrogen %	L-lysine HCl %	L-threonine %	Growth rate g/day
A	Basal (which contains 2.63 % roller-dried skim milk)	2 27	0.51	0.39	1.87 ± 0 06
B	As A, but with a total of 10 5 % roller dried skim milk	2 51	0.76	0 48	2.70 ± 0.08
C	As A, but with a total of 21.0 % roller dried skim milk.	2 79	1 00	0 55	3.04 ± 0.04
D	As A, but with a total of 26 3 %, roller dried skim milk	2 87	1.05	0 57	3.33 ± 0 04
E	As A, but with 0 55 % L-lysine HCl	2.27	0 90	0 37	3 13 ± 0.03
F	As A, but with 0.55 % L-lysine HCl and 0.22 % DL-threonine	2.51	0.93	0.44	3 06 ± 0.03

In per cent of the dry weight of the diet.
This amount of roller-dried skim milk corresponds to the 50 g 1 water in the dough which was always used. For the groups B, C and D the amount of dry milk corresponds to 200, 400 and 500 g/l
Standard error of the mean.
The loss of free L-threonine during the hydrolysis preceding the microbiological determination has been found to be of the order of 25—30 % (Eriksson, Larsson and Lee 1961).

The second series was carried out in a different animal room having wire bottom cages, a temperature of 25 C and a relative humidity of approximately 50 %. The rats for this experiment were Sprague-Dawley males and had a weight of 60 g at the time when they were given the experimental diets.

In both series the diets were fed *ad libitum*. The rats were given a non-supplemented bread diet for a few days before receiving the experimental diets. This was done mainly in order to accustom the rats to bread diets.

Results

Due to difficulties in obtaining rats suitable for growth rate experiments, the first series of experiments reported here had to be performed with rats having a higher initial weight than those used in the second series (see above). This influences the absolute values of the growth rates obtained. Since this should not, however, have any influence on the problem under study — viz. a comparison of the supplementary values of protein and free amino acids — the results of this series are presented although a direct comparison with the data of the second series of experiments is not possible.

Table II Composition of diets, growth rates and nitrogen efficiency ratios for the groups of rats i Series II Ten rats per group were fed the different diets for 21 days. All additions were made before baking

Group	Diet Additions listed below are expressed in per cent of the weight of the flour	Determined content of Nitrogen %	Determined content of L-lysine HCl %	Average gain in weight g	Average gain in weight per g nitrogen consumed g/g
A	Basal (which contained 2.70 % roller-dried skim milk)	2.21	0.55	36.7	8.4 ± 0.1
B	Basal + 0.22 % L-lysine HCl.	2.26	0.80	71.2	12.8 ± 0.3
C	Basal + 0.44 % L-lysine HCl	2.29	0.95	102.5	15.3 ± 0.3
D	Basal + 5.80 % roller-dried skim milk.	2.39	0 74	54 2	9.9 ± 0.2
E	Basal + 11.6 % roller-dried skim milk.	2.59	0.81	69.6	10.8 ± 0 3
F	Basal + 1.95 % fish protein	2.44	0.70	66.1	11 1 ± 0 2
G	Basal + 3.90 % fish protein	2.62	0.82	97.5	13.2 ± 0.2
H	Basal + 6.00 % soya bean protein	2.84	0.79	105.2	12.6 ± 0.2

In per cent of the dry weight of the diet.
Standard error of the mean.

Six groups of rats, originally comprising 12 animals each, were used in the first series of experiments. The first group was given the basal bread diet (which contains 50 g roller-dried skim milk per l of water in the dough) the second third and fourth groups diets with bread containing a total of 200, 400 and 500 g respectively of roller-dried skim milk per l of water in the dough, the fifth group a diet with a bread containing 0.55 % L-lysine HCl of the flour weight, and the sixth group a diet with a bread containing 0.55 % L-lysine HCl and 0 22 % DL-threonine. The amounts of roller-dried skim milk used in the second, third and fourth groups correspond to a total of 10 5 21 0 and 26 3 % of the fresh weight of the flour The contents in the diets of nitrogen, L-lysine HCl and L-threonine were determined and are shown in Table I

Six rats per group were killed after 21 days and the remainder after 49 days. Determinations of liver fat and histological examinations of the livers were carried out in all cases. The results of these observations together with those from other rat experiments will be presented in a forthcoming paper (LARSSON, RUBARTH and ERICSON 1962)

The average growth rates of the various groups are given in Table I It can be seen that a progressively increasing rate of growth resulted from the addition of increasing amounts of roller-dried skim milk to the dough. The

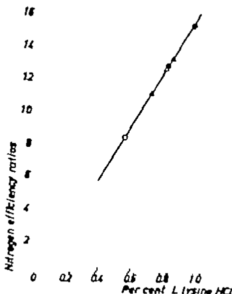


Fig. 1. Nitrogen efficiency ratios for the groups of rats in Series II as function of the content of L-lysine HCl in the diets.
○ Group A, ● group B, ● group C, group D group E, ▲ group F ▲ group G and △ group H.

effect of the addition of L-lysine HCl alone (group E) was slightly larger than the effect of the addition of 400 g roller-dried skim milk (group C) although the latter group supplied a greater amount of lysine (expressed as L-lysine HCl) The roller-dried skim milk provided in addition a significant quantity of threonine — the second most limiting amino acid in wheat bread (ERICSON 1960) — but it is obvious that this was without effect. This observation indicated that there was a considerable loss of the lysine in the roller dried skim milk during the baking an inference that is supported by a comparison of the groups D and F Group D in which the added lysine and threonine came from roller-dried skim milk, supplied more of both these two amino acids than group F Despite this, group F was clearly superior to group D

In the second series of rat experiments, 8 groups of rats each comprising 10 animals were used. The first group received a basal bread diet and the subsequent seven groups were provided with diets made from breads supplemented with two levels of free L-lysine HCl, two levels of roller-dried skim milk, two levels of fish protein and one level of soya bean protein as shown in Table II This table also gives the nitrogen and lysine (expressed as L-lysine HCl) contents of the diets, the average gain of weight and nitrogen efficiency ratios of the rats in the different groups The rats were given the various experimental diets for 21 days.

What stands out primarily from examination of the data in Table II is that, in comparison with the other supplements, the addition of roller-dried

skim milk failed to promote growth and nitrogen efficiency ratios in proportion to the lysine content of the corresponding diets. For the other supplements, a good correlation between the lysine content of the diet, as determined microbiologically and the nitrogen efficiency ratio is indicated. This becomes more obvious when the nitrogen efficiency ratios of the different groups are plotted as a function of the lysine content of the corresponding diets (Fig. 1). For all groups, with the exception of the two groups of rats that received diets containing additional roller-dried skim milk, the nitrogen efficiency ratios were directly proportional to the total lysine content of the diets regardless of whether the lysine had been supplied free or protein-bound. The groups given the diets fortified with dry milk had nitrogen efficiency ratios that were less than expected from the lysine content of the diets.

Discussion

The data obtained in the present investigation demonstrate that the improvement of the protein value of wheat bread resulting from the addition of various supplements is determined by the content of available lysine in the final product, as long as lysine remains the most limiting amino acid. The relationship appears to hold independently of whether the supplementary lysine is provided by lysine-rich proteins or in the form of L-lysine HCl. The same conclusion has been reached and explicitly stated by HOWARD et al. (1958), BROWN et al. (1959) and GUGGENHEIM and FRIEDMANN (1960) and is apparent also from the work of LIGHT and FREY (1943), HUNDLEY, ING and KRAUSS (1956), SABISTON and KENNEDY (1957), HUTCHINSON, MORAN and PACE (1959) and SCHWARTZ, TAYLOR and FISHER (1959). DESHPANDE, HARPER and ELVEHJEM (1957), on the other hand, state that "the finding that a mixture of essential amino acids simulating fibrin was not able to support as good a growth as the intact protein indicates some superiority of intact protein over amino acids as a dietary supplement (to wheat flour) under the conditions of the present experiments".

The assumption that free lysine and protein bound lysine are nutritionally equivalent and that no effect results from amino acids other than the most limiting supplied by the protein is implicit in the numerous attempts that have been made to determine the lysine availability in various foods (cf. SCHWEIGERT and GUTHNECK 1953, CARPENTER et al. 1957, KRATZER and GREEN 1957, THEN, JOHNSON and LIENER 1957, MAURON and MOTTU 1958, GUPTA et al. 1958, OLTERSHAUT, GRAU and LUNDHOLM 1959, CALHOUN, HEPBURN and BRADLEY 1960, MUELENAERE and FELDMAN 1960 and HELLER et al. 1961). One factor that could make such determinations uncertain would be a substantially different rate of absorption of the free and the protein-bound lysine. Only limited data seem to be at hand on this point but LONGENECKER and HAUSE (1958) and SCHWARTZ, TAYLOR and FISHER (1959) have

studied the problem in dogs nd chicks and report that free and bou d)sine are absorbed at a similar rate Another factor infl encing comparisons of the apparent nutritive values of f ee and protein-bound amino cids e cir cumstance that the requiremer of an amino acid becomes greater w he protein (or amino acid) conte t of the diet is increased (cf GRA 194 A M QUIST 1949 KRATZER, WILLIA S and MARSHALL 1950, BIRD 1 SA 1954, BRESSANI and MERTZ 1 53 M NAVEP and HARPER 1959 M W et al. 1959 SWENDSEID HARRIS a d TUTTLE 1960) In the experiments reported here this phenomenon has app arer ly ot greatl affected he results, p o t due to the rather small increase in the protein cont n he diets esul from the protein supplements.

The correlation between protein value and lysine content d scussed bov and shown in Fig 1 would of course be valid only p to th l of lysine which satisfies the requirement of the animal urde tudy Abov this lev l one would expect to obtain a beneficial effect a fr t ec d m st limiting amino acid (in the case of wheat, threonine furnished upple mentary protein. A comprehensive study of the supplementation w th its two most limiting amino acids, i. e. lysine and threonine has beer per formed by ROSENBERG CULIK and ECKERT (1959) who presented interesting observations pertinent to this problem.

It should also be pointed out that a relationship like the one presented in Fig 1 would hold only if the content of lysine found pon microbiological determination or a constant fraction thereof were available to the rat. This seems to be true in the cases where L-lysine HCl, fish protein or soya protein were used as supplements but not when the bread was fortified with roller dried skim milk. Most probably the greater loss of lysine from roller-dried skim milk as indicated by the results of both Tables I and II is partly due to the high amount of sugar mainly lactose, in the milk. Since HENRICSON et al (1961) have found only small differences in the protein values of spray-dried and roller-dried skim milk from the Swedish Milk Products, Stockholm, it seems likely that the loss of lysine occurred during baking and not during the manufacture of the roller-dried skim milk.

From a practical point of view the choice between protein and amino acid supplementation depends on a number of factors. The content of the desired amino acid n the supplementary protein and the degree of inactivation of the protein-bound amino acid compared with the free amino acid during the preparation (g baking, cooking etc.) of th supplemented product as discussed here and in a previous paper (ERICSON et al. 1961) are of obvious importance for deciding the cost of the improvement. However other aspects such as the stability of the supplement during torage and the possibility of making the additions reproducibly and cheaply on a large scale and w thout impairing the taste odour colour and texture of the final product should be mentioned. The possibl risk of introducing "Imbalances" by the dditions

should also be considered although this will most likely gradually become a less serious problem as more knowledge accumulates.

In earlier work (ERICSON 1960) we found that the addition of 0.4 % of L-lysine HCl to a bread diet after baking improved the nitrogen efficiency ratio by 85 %. In the present study it was found that the same amount of L-lysine HCl added before baking resulted in an improvement of 82 %.

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From the Department of Physiology U iversity of Turku, Finland

Studies of the Effect of X Rays on the Glucuronide Synthesis and β-Glucuronidase Activity in the Duodenal Mucous Membrane of the Rat

By

K. HARTIALA, V NÄNTÖ and U K. RINNE

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Abstract

HARTIALA, K., V NÄNTÖ and U K. RINNE. *Studies of the effect of X-rays on the glucuronide synthesis and β-glucuronidase activity in the duodenal mucous membrane of the rat.* Acta physiol. scand. 1961 53 376—380 — In a series of previous experiments we have studied the effect of local X-ray irradiation on the glucuronide conjugation capacity and on the β-glucuronidase activity of th liver and gastric mucosa. According to these studies the sensitivity of these tissues towards ir radiation is different. These studies also show that the changes in the glucuronid formation capacity and the β-glucuronidase activity are not parallel which is taken as a further indication that the β-glucuronidase enzyme is not involved in the synthesis of glucuronide-conjugates. In the present work we have studied the glucuronide conjugation and β-glucuronidase activity of rat duodenal mucosa after local X-ray irradiation.

Materials and Methods

One hundred thirty-two male rats (Wistar weight range 180–220 g) were used in the experiments with 77 control animals. The local X-ray irradiation was performed in Nembutal anesthesia, the duodenum was exposed through a mid-line excision, and the animal protected with a 3 mm lead shield. The local X-ray irradiation was performed by single 400 r (group A) and 1,200 (group B) doses (185 kV X-ray machine, 10 mA, 0.5 cm Cu), as described previously (HARTIALA, NÄNTÖ and RINNE 1958, 1959 and HARTIALA *et al* 1960).

Prior to and after th operation and irradiation the animals were fed with usual laboratory diet and water ad libitum. All of the animals survived for the later analyses.

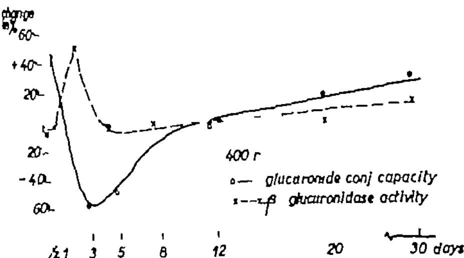


Fig. 1. Changes in duodenal glucuronide conjugation capacity and β-glucuronidase activity expressed as per cents at various times after local X-ray irradiation.

The animals were killed in 8 groups, 12 hours, and 1 3, 5, 8, 12, 20 and 30 days after the irradiation. Specimens were taken from the mucous membrane of the duodenum and the glucuronide synthesis measurements (according to LEVVY and STOREY 1949 HARTIALA and RONTU 1955) and β-glucuronidase assays (TALALAY FISHMAN and HUGGINS 1946, modified by FISHMAN, SPRINGER and BRUNETTI 1948) were performed from each animal. Duplicate determinations were made from each sample. In the statistical treatment of the results "Student's" t-test was used.

Table I. Effect of local X-ray irradiation on the glucuronide synthesis. The results expressed as μg of o-aminophenolglucuronide produced by the duodenal mucous membrane per 100 mg dry weight tissue

Time after irradiation	Group A (400)			Group B (1,200)		
	Number of animals	μg/100 mg	P*	Number of animals	μg/100 mg	P*
12 hours	12	119 ± 11.3	> 0.05	6	94 ± 4.8	> 0.05
1 day	12	189 ± 18.0	< 0.01	6	145 ± 10.2	> 0.05
3 days	6	54 ± 3.5	< 0.01	6	37 ± 3.8	< 0.001
5 days	12	67 ± 3.6	< 0.01	6	85 ± 13.6	> 0.05
8 days	6	110 ± 7.6	> 0.05	12	120 ± 21.1	> 0.05
12 days	12	123 ± 14.4	> 0.05	12	119 ± 12.0	> 0.05
20 days	6	148 ± 15.6	> 0.05	8	150 ± 15.0	> 0.05
30 days	6	165 ± 18.8	> 0.05	6	175 ± 18.9	> 0.05
Control animals	77	150 ± 6.4				

Standard error

P values refer to comparison with the control animals.

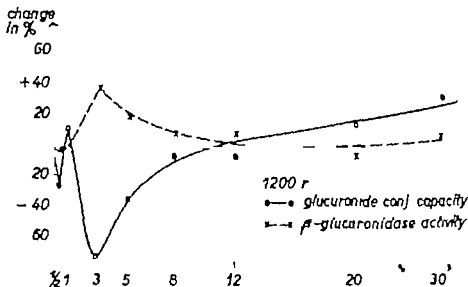


Fig. 2. Changes in duodenal glucuronide conjugation capacity and β-glucuronidase activity expressed as per cents at various times after local X-ray irradiation.

Results

Glucuronide formation (Table I Fig 1 and 2) Both after the 400 r and 1,200 r irradiation a slight decrease in the conjugation capacity was present 12 hours after the treatment. This was followed by an increase within the next 12 hours, (400 r $P < 0.01$) After this the conjugation capacity diminishes greatly so

able II Effect of local X-ray irradiation on the β-glucuronidase activity in the duodenal mucous mbrane The results expressed as β-glucuronidase units per g wet weight tissue

Time after irradiation	Group A (400)			Group B (1,200 r)		
	Number of animals	β-glucuronidase units g	P	Number of animals	β-glucuronidase units g	P
12 hours	12	1,341 ± 92	> 0.05	6	1,320 ± 208	> 0.05
1 day	12	1,364 ± 96	> 0.05	6	1,389 ± 166	> 0.05
3 days	6	2,033 ± 235	< 0.001	6	1,911 ± 282	< 0.01
5 days	12	1,313 ± 147	> 0.05	6	1,680 ± 167	> 0.05
8 days	6	1,381 ± 115	> 0.05	12	1,498 ± 59	> 0.05
12 days	12	1,411 66	> 0.05	12	1,329 ± 167	> 0.05
20 days	6	1,308 ± 195	> 0.05	6	1,297 ± 104	> 0.05
30 days	6	1,524 63	> 0.05	6	1,502 ± 41	> 0.05
Control animals	77	1,394 ± 45				

Standard error
P values refer to comparison with the control animals.

that the decrease is [illegible]3 per cent with the 400 r dose (P < 0.01) and [illegible]1 per cent with the 1,200 r dose (P < 0.001). The conjugation capacity approaches the control level within 8 days and 20 and 30 days after the irradiation it was above the control level. The difference, however, is not significant.

β-glucuronidase (Table II, Fig. 1 and 2). After a slight initial decrease 12 hours after the treatment the enzyme activity rises being at its maximum 3 days after the irradiation. The increase is 50 per cent with the 400 r dose (P < 0.001) and 5[illegible] per cent with the 1,200 r dose (P < 0.01). After this the activity settles down to the control level this being more rapid after the smaller irradiation dose. On the later periods no differences with the control values were noted.

Discussion

The duodenum is the most radio-sensitive part of the intestinal tract (DESJARDINS 1932, PIERCE 1948, LESHER 1957). In rats exposed to 600 [illegible] r of total-body X-irradiation, degenerative changes and cell death in the duodenal epithelium are seen 3 to 8 hours after the irradiation, and bizarre cells from 1 to 3 days. Recovery is very rapid: at 5 days very little residual effect is seen. The greatest damage is located to the basal cells of the crypts of Lieberkühn. The epithelium of the Brunner's glands is more resistant than the villous or crypt epithelium (PIERCE 1948).

Not much information is available of the radiation effects on the enzyme systems of the intestinal mucous membrane. FRENCH and WALL (1957) showed that rat intestinal cholinesterase activity was reduced 48 hours after irradiation. Absorption by the small intestine after irradiation is reported to be decreased (MEAD, DECKER and BENNETT 1951, BUCHWALD 1931). DICKSON (1933) observed no impairment of hexokinase activity after irradiation. MOSS (195[illegible]) estimated the total phosphorylation activity of the exteriorized X-irradiated small intestine, and found a decrease in phosphorylation. KAY and ENTENMAN (1959) measured the glucose oxidation rate of rat small intestine mucous membrane, and found an increase on the 3rd and 4th post-irradiation days. They concluded that this increase after X-irradiation is due entirely to increased citric acid cycle activity.

As in our previous studies local X-ray irradiation applied to the duodenal mucosa produces severe changes in the conjugation functions. After the initial stimulation of the glucuronide conjugation the following depression reaches its maximum on the third day and is about the same magnitude as observed in similar studies with the liver. The recovery takes about one week and is actually over-compensated as in the liver. The duodenal mucosa appears to be somewhat more sensitive towards the smaller radiation dosage as the liver.

As compared to the gastric mucosa the response to radiation appears to follow a different pattern. The most obvious difference is the slower recovery in the gastric mucosa.

As to the β-glucuronidase activity under the same conditions the changes

do not coincide with the changes in the glucuronide conjugation capacity. In this respect the results agree with our previous observations. On the other hand such great increase in the activity as was seen in the liver as a late effect was not present.

It would thus appear that the sensitivity of the studied tissues towards x-radiation is somewhat different. The different life span of the cellular elements in the various organs might explain these changes. On the other hand these studies do not reveal which part of the complex enzyme machinery in the glucuronide synthesis is involved in the observed changes. These studies are now under progress in this laboratory.

This study has been supported by a grant from the Sigrid Jusélius Stiftelse.

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Acta physiol. scand. 1961. 53. 381—386

From the Institut of Occupational Health, Työterveyslaitos, Helsinki, Finland

Stimulation of Sweating by Exercise after Heat Induced "Fatigue" of the Sweating Mechanism

By

K. Ahlman and M. J. Karvonen

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Abstract

Ahlman, K. and M. J. Karvonen. *Stimulation of sweating by exercise after heat induced fatigue of the sweating mechanism.* Acta physiol. scand. 1961. 53. 381—386. — When wrestlers lose weight before competition, they often induce sweating in a hot bath until the "fatigue" of the sweating mechanism. It is reported that at this stage exercise may still induce sweating. This claim was tested in experiments on five young males. After three or four exposures of 12 min each to ambient heat in "sauna" bath which caused the rectal temperature to rise to values in range from 38.4 to 39.4 C, substantial sweating could no more be elicited by repeating the thermal stimulus. However treadmill running for 20 min caused further weight loss of 0.1 to 0.9 kg, although the rectal temperature now remained on an average 0.7 C lower than during the preceding exposure to heat. The mean total loss of weight during the whole procedure was 3.6 per cent of the initial body weight.

The concentration of sodium, potassium and chloride varied individually at repeated exposures to heat. In most subjects, the range of the variation was small. During exercise the mean content of sodium was lower than in sweat collected during the first exposure to ambient heat.

The results are discussed.

When wrestlers have to lose weight before a competition, they induce sweating in a hot bath or through cross-country running. A prolonged exposure to heat causes an apparent fatigue of the sweating mechanism. It is known among wrestlers that at this stage, further sweating may still be induced by exercise, e. g. by cross-country running (Ahlman and Karvonen 1961). This phenomenon does not appear to be previously mentioned in the literature on sweating.

An experimental study was made, imitating the method of losing weight as used by wrestlers. Attention was paid to weight loss, rectal temperature and the mineral composition of sweat.



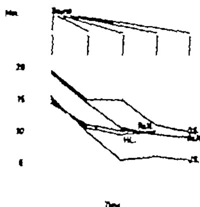


Fig. 1

Fig. 2

Fig. 1. The loss of body weight at repeated exposures to ambient heat in the sauna. After heat induced sweating had stopped the subjects ran for 20 min on treadmill.

Fig. 2. The duration of visible sweating after repeated exposures to heat.

Method

Five male students of physical education served as subjects. The experiment was started by weighing the subject, after he had emptied the bladder and defecated. The entire skin was thoroughly washed and dried. The right arm was enclosed in a plastic sleeve, and the rectal temperature was recorded. The subject was then exposed to intense heat in Finnish "sauna" bath for 12 min. The rectal temperature was recorded after 9 min in the sauna.

On leaving "sauna" the volume of sweat in the plastic sleeve was measured, and a sample was taken for mineral analysis. The subject was wrapped in blankets, in order to retard the fall of body temperature. He remained supine at ordinary room temperature as long as visible sweating continued. When it had ended, the skin was again washed, dried, and the subject was weighed.

The above procedure was repeated three or four times, i. e. as long as significant sweating could be elicited.

After the last exposure to ambient heat, the subject ran on treadmill for 20 min, during which sweat was again collected. At the termination of running, the rectal temperature and body weight were again recorded.

The chloride concentration in sweat was determined by using the method of SCHALES and SCHALES (1941) as modified by BRUN (1949) and the sodium and potassium with flame photometer using lithium as an internal standard.

Results

The total loss of weight varied from 1.5 to 3.0 kg. The amount of weight lost at each stage is shown in Fig. 1. Most weight was lost during the first exposure to heat. The mean losses at each exposure were 1.6, 0.7, 0.5, 0.1 and 0.0 per cent of the initial body weight. The "fatigue" of the sweating mechanism was seen also in a shortening of the duration of visible sweating after each exposure (Fig. 2).

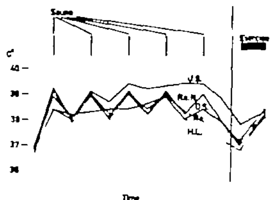


Fig 3. Rectal temperature in sauna, during subsequent rest periods, and before and after treadmill running.

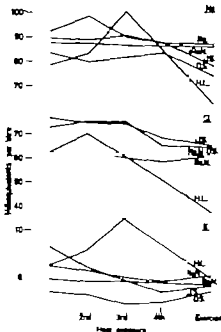


Fig 4. Sweat potassium, sodium and chloride content during repeated exposures to ambient heat and subsequent exercise.

The rectal temperature offered no explanation to the diminishing returns of sweat t remained essentially constant at each exposure, or showed a tendency to a rise (Fig 3) This pplied both t temperature in sauna and during the subsequent sweating at room temperature.

In all subjects, running agai induced sweating The loss of weight aried from 0 1 to 0.9 kg The mean loss was 0 6 per cent of the initial body weight. This occurred while the rectal temperature remained essentially lower than during the exposure to ambient heat.

Table I Sweat sodium, potassium and chloride during first exposure to heat and during exercise

Electrolyt	Milliequivalents per litre		t	P
	Heat	Exercise		
Sodium	86.0	76.8	3.05	0.05
Potassium	6.62	5.53	1.31	
Chloride	67.0	57.8	2.05	

The variations of the electrolyte content of sweat are shown in Fig 4 Considerable individual variations are seen. They are, however less marked for chloride than for sodium and potassium. In one subject, H L. the sodium and potassium show a steep rise. In this subject, sweating stopped early and the total weight loss was smallest, 1.5 kg only

In sweat collected during exercise, the mean content of each electrolyte was lower than in that secreted during the first stay in the sauna. However the difference was significant only for sodium ($P < 0.05$ Table I) When the electrolyte content of the work sweat is compared with that in the immediately preceding collection in sauna, sodium again shows a consequent fall, whereas potassium and chloride show no regular tendency

Discussion

A fatigue of the sweating mechanism may be produced either by prolonged and copious thermal sweating (HANCOCK *et al.* 1929 LADELL 1945, GERKING and ROBINSON 1946) or by repeated administration of drugs like metacholine (THAYSEN and SCHWARTZ 1955) In the present experiments, after sweating could no more be induced through repeated exposures to intense heat, exercise still was able to stimulate copious sweating in all subjects. This occurred, while the rectal temperature was on an average 0.7° C lower than during the preceding exposure to heat. Either the sweat glands rapidly recovered from the exhaustion, or the "fatigue" of the sweating mechanism was not at all due to the exhaustion of the glands.

The skin temperature is markedly different under the two conditions In the sauna the mean skin temperature may rise to 42° C (PIIRONEN and ÄIKÄS 1960) while at exercise it obviously does not exceed body temperature. According to ROBINSON and GERKING (1947) the sweating mechanism is "fatigued" the sooner the higher the skin temperature. With this observation, the present results are in an agreement.

The sodium and chloride concentration of thermal sweat is known to increase, as sweating becomes more profuse (KITTSTEINER 1911 1913 DILL *et al.* 1938, BÖTTNER and SCHLEGEL 1940 KYUTOKU 1940, CHUN 1942, LOCKE *et al.* 1951) On the other hand the sodium and chloride content of sweat is reported

to rise progressively as fatigue develops durin_ prolonged thermal sweating (Robinson and Robinson 1954 In the present series, however the sodium and chloride concentration rema ned essentially unchanged or slightly decreased with repeated exposures In successive samples of sw t taken during an exposure to heat in the sau th sodium and chloride concentration do not change, whereas the potass um conc ntration decreases (Ahlman et al 1953). In this respect the situat on resembl les that d e to repeated injections of metacholine a "fatigue of the weating mechanism ensues, w thout any change of the sodium content Dole et al 1951

The sweat sod'um and chloride concentration is eported also to increase with the skin temperature (Kittsteiner 1913 Lehmann and Szakall 1939 Johnson et al. 1944 Ladell 1945 Robinson et al 1950, Weiner and van Heyningen 1952) The tendency to a lo er sodium concentration n the extreme tests — with lower skin temperat re — ir rda ce with this finding. However when sweating ha been induced d t da either by heat or exercise, no regular differences ha e been observed in the electrolyte composition (Hasan et al. 1954)

The expenses of the work have been covered by grant from the State Athletic Board.

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to rise progressively as "fatigue" develops during prolonged thermal sweating (ROBINSON and ROBINSON 1954). In the present series, however, the sodium and chloride concentration remained essentially unchanged or slightly decreased with repeated exposures. In successive samples of sweat taken during an exposure to heat in the "sauna" the sodium and chloride concentration do not change, whereas the potassium concentration decreases (AHLMAN *et al.* 1953). In this respect the situation resembles that due to repeated injections of metacholine: a "fatigue" of the sweating mechanism ensues, without any change of the sodium content (DOLE *et al.* 1951).

The sweat sodium and chloride concentration is reported also to increase with the skin temperature (KITTSTEINER 1913, LEHMANN and SZAKALL 1939, JOHNSON *et al.* 1944, LADELL 1945, ROBINSON *et al.* 1950, WEINER and VAN HEYNINGEN 1952). The tendency to a lower sodium concentration in the exercise tests — with lower skin temperature — is in accordance with this finding. However, when sweating has been induced on different days either by heat or exercise, no regular differences have been observed in the electrolyte composition (HARAN *et al.* 1954).

The expenses of the work have been covered by a grant from the State Athletic Board.

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